Influence of feed formulation on properties of marine fish farm effluents.

James, Jack Morgan

Jack Morgan James, B.Sc. (Hons)

Influence of feed formulation on properties of marine fish farm effluents

Submitted to the University of Wales in fulfilment of the requirements for the Degree of Doctor of Philosophy

Swansea University

2009

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Summary

The replacement of fishmeal by alternative protein sources in feeds for farmed fish is important in the context of limited global availability and high price of industrial fishmeal. However, little is known about the effects of novel protein sources on the effluents produced by aquaculture operations. Controlled feeding experiments were therefore conducted using Atlantic cod (*Gadus morhua*) and European sea bass (*Dicentrarchus labrax*) offered feeds in which up to 35% by weight of the fishmeal was replaced by one of 7 different or differently processed protein sources. All plant proteins were found to be acceptable at the levels tested, without any negative impacts on growth or gastrointestinal tract histo-pathology in either species. Analyses of faecal particle size distribution, zeta potential (ζ – particle surface charge characteristics) and rheology (fluid behaviour) were used to determine the physical characteristics of faeces from fish receiving these experimental feeds.

Faeces produced by both fish species when fed experimental feeds contained a lower proportion of fine particles compared to fishmeal controls. However, when the actual volume of faeces was accounted for, most experimental feeds resulted in a greater volume of fine particles due to the higher total production of faeces. ζ analysis showed that all faecal samples are negatively charged at system pH, suggesting that there will be electrostatic repulsion under standard rearing conditions. The charge characteristic could be exploited by the addition of oppositely charged flocculants into the feeds; however, the incorporation of blood meal in feeds was not successful in accomplishing this. Rheological analysis demonstrated that the addition of plant proteins into the feeds increased the viscosity and stability of the faeces. Areas of further work are identified, including refinement of rheological methods and the investigation of more suitable flocculant additives, to help reduce the impacts of effluents on rearing systems and the environment.

Declaration

This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

Signed..... ..... (candidate)

Date.....23/09/09.....

Statement 1

This thesis is the result of my own investigations, except where otherwise stated. Where correction services have been used, the extent and nature of the correction is clearly marked in a footnote(s).

Other sources are acknowledged by footnotes giving explicit references. A bibliography is appended.

Signed..... ..... (candidate)

Date.....23/09/09.....

Statement 2

I hereby give consent for my thesis, if accepted, to be available for photocopying and for inter-library loan, and for the title and summary to be made available to outside organisations.

Signed..... (candidate)

Date.....23/09/09.....

Contents

Acknowledgements

I would like to acknowledge the following people and organisations who have helped me along the way in designing and carrying out my research, in writing and preparing the thesis for submission, and in supporting me financially and personally. Thanks to Skretting UK for providing financial support and to Skretting ARC for feed production and analyses, with particular thanks to Paul Morris and Vibeke Vikeså for their help and guidance. Thanks also to Rhodri Williams via the EPSRC Complex Fluids Portfolio Partnership and to the School of Environment and Society in providing financial support. Thank you to my supervisors Robin Shields and Bob Lovitt, for their advice, guidance and constructive criticism. A special thank you to the staff and students at the Centre for Sustainable Aquaculture Research, who have helped immeasurably in husbandry and sampling throughout the research. Thanks to Ingrid Lupatsch for her advice and practical help, and to Jon Buckley, Leigh Castleman and Janielle Wallace for use of data from their MSc. dissertations. For their help in training me on various instruments and in making sense of the results my sincere thanks go out to Rhodri Williams, Paul Williams and Matthew Lawrence in the School of Engineering. And finally, thanks to my family and friends who have kept me grounded and who have supported and encouraged me through some challenging times in the course of carrying out this PhD, no one excluded.

List of Tables

List of Figures

List of Abbreviations

µl	-	Microlitre
µm	-	Micrometre
1/s	-	Shear rate
ANOVA	-	Analysis of variance
–COOH	-	Carboxyl group
CSAR	-	Centre for Sustainable Aquaculture Research, Swansea, University
DM	-	Dry matter
FCR	-	Feed conversion ratio
g	-	Gram
g	-	Gravity
G'	-	Storage modulus (elastic modulus)
G''	-	Loss modulus (viscous modulus)
GI	-	Gastrointestinal
GLM	-	General linear model
HSI	-	Hepatosomatic index
hr	-	Hours
Hz	-	Hertz (frequency)
IEP	-	Isoelectric point
kg	-	Kilogram
KW	-	Kruskal Wallis test
l	-	Litre
m^3	-	Cubic metres
min	-	Minutes
ml	-	Millilitre
mm	-	Millimetre
mV	-	Millivolts
$–NH_2$	-	Amino group
nm	-	Nanometre
OM	-	Organic matter
Pa	-	Pascals (stress)

Pa.S	-	Pascal seconds (complex viscosity)
pK_a	-	Acidity constant
pK_b	-	Basicity constant
ppm	-	Parts per million (concentration)
ppt	-	Parts per thousand (concentration)
RAS	-	Recirculating aquaculture system
s	-	Second
SD	-	Standard deviation
SGR	-	Specific growth weight
TSS	-	Total suspended solids
UV	-	Ultraviolet
Uε	-	Electrophoretic mobility
ζ	-	Zeta potential

Chapter 1

General Introduction

1.1 Fish meal replacement in aqua feeds

The finfish and crustacean aquaculture sector is currently growing at 8.5% a year, producing over 37 million tonnes in 2006, 56.3% of total aquaculture production (Tacon and Metian, 2008). It can be expected that the growth in the demand for nutrient inputs to the sector, in terms of formulated feeds, will increase in line with this increase in production. Naturally, the aquaculture industry relies heavily on fish meals and oils derived from targeted capture fisheries to provide the basis of these commercial formulated aqua feeds.

In 2006, the aquaculture industry consumed 68.2% of world fish meal supplies and 88.5% of world fish oil production, an approximately twofold increase in both nutrient sources from 1995 (Tacon and Metian, 2008). These are in contrast to predicted values for 2010 made in 2001/2 of 56 to 60% of global fish meal supplies and 92 to 97% of global fish oil production (Anon, 2001, Sabaut, 2002). These figures show quite clearly that the impact of aquaculture on wild pelagic fish stocks is marked, and will continue to be so as the industry continues to expand. Fish products are also the most expensive constituent of modern aqua feeds, with fish meal reaching $1046 per tonne in February 2009, down from a high of $1297 a tonne in September 2006 but up 106% from $508 per tonne in February 1999 (Barrientos, 2009). Fish oil has undergone an even more extreme increase in price, up approximately 143% to $1700 per tonne in early 2008, from $700 per tonne in 1998 (Tacon and Metian, 2008). The replacement of fish meal and oil by other protein and lipid sources is therefore important in terms of both ecological and economical sustainability. Furthermore, current catch quotas set by governmental organisations in many areas (Pike and Barlow, 2002), while essential in maintaining stocks for the future, will serve to limit the volume of material available to supply the demand.

In order to reduce the pressure on wild meal fish stocks and to improve the long term sustainability of the aquaculture industry, there is currently a major focus on the potential of plant proteins to replace a proportion of the protein supplied by fish meal in aqua feeds. Such research, coupled with pressures resulting from static or diminishing wild fish stocks, reduced fish meal availability, increasing price of meal fish due to increasing costs associated with capture, and heightening market pressure

to improve sustainability, have led to a reduction in the average dietary fish meal level in formulated feeds for marine fish from 50 to 32% between 1995 and 2005 (Tacon and Metian, 2008). Indeed, the use of fish meal by the aquaculture industry is set to continue to decrease in the long term for these reasons, resulting in an increased demand for plant proteins.

Feed ingestion, digestion or nutrient utilisation can potentially be negatively impacted by novel protein sources, affecting their suitability as fish meal protein replacers. Feed palatability is a major factor affecting ingestion; however due to most feeds containing at least a proportion of fish meal, in most cases this does not appear to be a significant obstacle. The utilisation of protein in the novel sources is generally measured in terms of growth performance in fish nutrition studies. Leading into this and therefore one of the most important aspects to measure is the bioavailability of protein to the animals (its digestibility), as well as that of lipid, dry matter and organic matter (Drew *et al.*, 2007).

A reduction in fish growth performance as a result of plant protein inclusion can result from alterations to the digestive tract of the experimental fish, as described by Baeverford and Krogdahl (1996). They described pathological changes in the mucosal lining of the distal intestine in Atlantic salmon (*Salmo salar*), classified as non-infectious subacute enteritis, caused by factors in the feeds introduced by the substitution of plant proteins for fish meal, for example trypsin inhibitor in soybean meal (soybean meal) (Friedman *et al.*, 1991, Francis *et al.*, 2001, Friedman and Brandon, 2001). However, in general it is found that proper processing of meals, for example by heat and protein extraction, can inactivate the usually labile antinutritional factors, and result in feeds that are safe to use in those species susceptible to enteritis (Refstie *et al.*, 2001, Drew *et al.*, 2007), even at full replacement of fish meal (Escaffre *et al.*, 2007).

Despite some negative impacts of plant proteins in feeds for farmed fish, in general there have been very promising results from using a range of plant protein sources in feeds for numerous marine and freshwater fish species. It is however important that research continues to be carried out on as many of the species of fish farmed today as possible, in order to ensure the suitability of particular protein sources for particular

species. Moreover, such research will allow the upper limits of plant protein inclusion to be ascertained, particularly pertinent in the current climate of rising fish meal prices and market awareness of sustainability issues.

1.1.1 Soy

Soybean (*Glycine max*) as an alternative protein source is one of the most widely trialled, having been tested on numerous species of marine and freshwater fish. In general, two forms of soybean protein are used in the production of aqua feeds, those being soybean meal which is the ground beans with limited processing, and the soy protein concentrate (soy conc.) which is the extracted protein. Soybean meal has been tested in several different forms and studies have shown that the method of processing the raw material can have significant effects on the quality of the finished product and its effectiveness as a fish meal substitute.

Boonyaratpalin *et al.* (1998) substituted 15% fish meal by weight with four differently processed soybean meals in feeds for Asian sea bass (*Lates calcarifer).* They demonstrated that the growth rate of fish fed soaked raw full fat soybean meal was consistently and significantly lower than that for solvent-extracted, extruded full-fat and steamed full-fat soybean meal. Mortality was also high in the fish fed soaked raw full fat soybean meal and feed efficiency ratio and protein digestibility was low. There was however a reduction in feed intake among all the soybean meal feeds suggesting low palatability, and it was concluded that if palatability were to be improved fish meal could be replaced in part by properly processed soybean meal in feeds for *L. calcarifer.* The poor utilisation of raw full fat soybean meal was suggested to be due to high levels of trypsin inhibitor (an antinutritional factor). The high level of trypsin inhibitor found in this feed was probably due to the lack of heating or solvent processing. Heat processing is known to inactivate the Bowman-Birk and Kunitz inhibitors of chymotrypsin and trypsin with an inactivation efficiency of 80% to nearly 100% depending on the soy strain and the type of processing used (Friedman *et al.*, 1991, Francis *et al.*, 2001, Friedman and Brandon, 2001).

Adequately processed soybean meal has also been shown to effectively replace between 15% and 40% fish meal in feeds for olive flounder (*Paralichthys olivaceus),*

Atlantic halibut (*Hippoglossus hippoglossus*), Australian shortfinned eel (*Anguilla australis australis*), Atlantic cod (*Gadus morhua*), Atlantic salmon (*Salmo salar*), Nile tilapia (*Oreochromis niloticus*), and gilthead sea bream (*Sparus aurata*) resulting in comparable growth to the control (Kikuchi, 1998, Carter and Hauler, 2000, Grisdale-Helland *et al.*, 2002, Schneider *et al.*, 2004, Engin and Carter, 2005, Hansen *et al.*, 2007, Bonaldo *et al.*, 2008). Choi *et al.* (2004) demonstrated that dehulled soybean meal could replace 20% fish meal without any additives, or 30% with amino acid and/or attractant additives in feeds for *P. olivaceus*, while at 40% growth was retarded.

Comparable digestibility to a control feed at up to 30% soybean meal inclusion was also recorded in feeds for haddock (*Melanogrammus aeglefinus)*, rainbow trout (*Oncorhynchus mykiss), S. salar, A. australis australis*, Coho salmon (*Oncorhynchus kisutch*) and silver perch (*Bidyanus bidyanus*) (Boonyaratpalin *et al.*, 1998, Sugiura *et al.*, 1998, Allan *et al.*, 2000, Engin and Carter, 2002, Glencross *et al.*, 2004, Tibbetts *et al.*, 2004, Glencross *et al.*, 2005). Up to 15% inclusion also did not affect proximate body composition of *O. niloticus* (Schneider *et al.*, 2004), *G. morhua* up to 16% (Hansen *et al.*, 2007), or *P. olivaceus* and *O. mykiss* up to 40% inclusion (Kikuchi, 1998, Choi *et al.*, 2004, D'Souza *et al.*, 2006).

In terms of soy conc., Day and Gonzalez (2000) found that in feeds for *Psetta maxima*, replacing 25% fish meal with soy conc. showed no significant difference in SGR from the control. Levels of around 30% inclusion have also shown to result in adequate digestibility in experiments involving *G. morhua*, turbot (*Scopthalmus maximus* or *Psetta maxima*), *Hippoglossus hippoglossus*, *O. mykiss* and *S. salar* (Berge *et al.*, 1999, Day and Gonzalez, 2000, Glencross *et al.*, 2004, Glencross *et al.*, 2005, Tibbetts *et al.*, 2006). In the case of sterlet (*Acipenser ruthensus*) 100% replacement of fish meal by soy conc. resulted in no significant differences in digestibility from the control (Ustaoglu and Rennert, 2002). Significant differences have however been noted in whole body proximate composition, with 28% inclusion resulting in an increase in dry matter, and 15% resulting in a significant reduction in ash in feeds for *H. hippoglossus* and *O. niloticus* respectively (Berge *et al.*, 1999, Schneider *et al.*, 2004).

It therefore appears from the literature quoted that appropriately treated soybean proteins can replace around one quarter of fish meal proteins in aqua feeds for marine cultivated fish. This demonstrates clearly the need to determine the effects of plant proteins in different species, as there appears to be differing degrees of flexibility in accepting new proteins between species. Hind gut histological alterations in salmonids (discussed subsequently) and slightly lower digestibility values in some species have led some authors to suggest that soybean meal might be substituted with properly treated lupin meal as a safer alternative (Robaina *et al.*, 1995).

1.1.2 Lupin

Lupin seed meal has been shown in numerous studies to be a very promising fish meal substitute in aquaculture feeds. Glencross *et al.* (2002) found that a 50% inclusion of *Lupinus luteus* resulted in significantly enhanced growth over a fish meal control, and in another that 30% *Lupinus angustifolius* performed as well as the control (Glencross *et al.*, 2008) both in feeds for *O. mykiss*. In the latter they did not test a 50% inclusion.

A study using juvenile *P. maxima* found that extruded lupin meal could be incorporated into feeds at up to 50% with no adverse effects on growth performance, despite an initial depression of intake up to three weeks into the trial with increasing lupin incorporation (Burel *et al.*, 2000a). Lupin meal was found to result in one of the highest protein digestibility values at 30% inclusion for *A. australis australis*, *B. bidyanus, O. mykiss* (significantly higher than fish meal) and *P. maxima* (Allan *et al.*, 2000, Burel *et al.*, 2000b, Engin and Carter, 2002). In other trials with *O. mykiss*, lupin meal included at up to 70% increased the protein digestibility of feeds over fish meal, with a significant increase in whole body dry matter at 30% inclusion (Burel *et al.*, 1998). Similar results were seen in trials involving *S. aurata* by Robaina *et al.* (1995) who showed that lupin meal resulted in enhanced protein digestibility over soybean meal, with the plant proteins making up 30% of the feed.

A study by Glencross and Hawkins (2004) compared different species of lupin as protein sources, specifically *L. angustifolius, L. albus* and *L. luteus* in feeds for *P. auratus*. Protein digestibility was more than 95% in all cases, with *L. albus* showing

the highest value. However *L. luteus* demonstrated relatively high protein content and overall high levels of digestibility, and was suggested as the most promising substitute. This study illustrates that there can be substantial intra-genus differences in nutrient content and digestibility between plant species, and that it must not be assumed that one species would be as suitable as another within the same genus. A further study on *S. aurata* by Pereira & Olivia-Teles (2004) evaluated *L. angustifolius* in both raw and micronised (infrared treated) seed meal protein forms. Growth rate was significantly higher among fish fed the micronised meal rather than the corresponding raw meal. The same was seen with 20% micronised lupin protein, resulting in significantly higher growth rate than the control. The authors concluded that both types of protein were suitable for replacement of up to 30% fish meal protein without significant negative impact and that the process of micronisation significantly improved the dietary value of lupin meals. The authors also found no changes in whole body proximate composition between any feeds.

These studies indicate that lupin proteins can realistically replace between 20 and 30% of fish meal proteins in aqua feeds for marine fish species, similar to the potential replacement levels for soybean. However, there may be more scope for lupin inclusion due to the reduced need for heat processing prior to use and the extremely low levels of protease inhibitors (Drew *et al.*, 2007).

1.1.3 Wheat gluten

Wheat gluten has been shown to be another potentially good source of dietary protein for fish, comparing favourably with fish meal in terms of growth for *O. niloticus* at 15% inclusion (Schneider *et al.*, 2004). At complete substitution, Kissil and Luptasch (2004) found that wheat gluten resulted in a significant increase in growth rate in *S. aurata* compared to fish meal, corn gluten and soybean meal at complete substitution.

Robaina *et al.* (1999) found that the digestibility of feeds containing 30% wheat gluten was higher than fish meal feeds. Glencross and Hawkins (2004) reported that wheat gluten had a higher digestible protein level than most tested lupins and soya. In other studies, wheat gluten exhibited equivalent digestibility to fish meal up to a 30% inclusion level in feeds for *B. bidyanus, O. kisutch* and *O. mykiss* (Sugiura *et*

al., 1998, Allan *et al.*, 2000) and the highest digestibility out of a range of plant proteins for *G. morhua* (Tibbetts *et al.*, 2006). In terms of whole body proximate composition, Schneider *at al.* (2004) reported a significant reduction in ash in *O. niloticus* fed wheat gluten at 15% inclusion level. Published evidence therefore indicates scope for relatively high inclusion of wheat gluten in feeds for farmed fish, due to high digestibility and perhaps because of its purity (absence of antinutrional factors).

1.1.4 Faba beans

Faba bean (*Vicia faba*) meal as a protein source has only recently been investigated in any detail in feeds for farmed finfish. Gaber (2006) studied the effects of replacing 25%, 50%, 75% and 100% of the fish meal in feeds for *O. niloticus* fry, showing that up to 50% of fish meal (25% total inclusion) could be replaced in such feeds without affecting growth or digestibility. Similarly, juvenile *O. niloticus* were found to have comparable FCR, growth rate and digestibility on feeds with up to 24% total faba bean inclusion replacing soybean meal, while at 36% these parameters were significantly depressed (Azaza *et al.*, 2009). Allan *et al.* (2000) however did not detect any significant differences in protein digestibility for *B. bidyanus* receiving faba bean versus fish meal at 30% inclusion rate. In the case of *D. labrax*, faba bean inclusion at up to 30% significantly enhanced protein digestibility (Adamidou *et al.*, 1999).

Certain studies have also investigated the potential for differences between the digestibility of whole faba beans and dehulled faba beans. Booth *et al.* (2001) directly compared whole faba beans and dehulled faba beans at 30% substitution in feeds for *B. bidyanus* and found that dehulling significantly improved protein digestibility. Aslaksen *et al.* (2007) also studied whole versus dehulled faba beans in feeds for *S. salar*, finding no significant differences in digestibility compared to fish meal at 22% and 19% inclusion respectively. It appears from these studies and those mentioned previously that faba bean can be included successfully at up to 25%, and in particular following dehulling.

1.1.5 Fish health implications of plant protein inclusion

Studies involving salmonids have showed that these fish demonstrate a rapid onset of severe enteritis even at relatively low levels of plant proteins, in particular soybean meal's (Baeverfjord and Krogdahl, 1996, Refstie *et al.*, 2000, Refstie *et al.*, 2001, Krogdahl *et al.*, 2003, Aslaksen *et al.*, 2007, Uran *et al.*, 2008, Romarheim *et al.*, 2008a). In contrast to these reports, enteritis is rare in studies involving plant protein substitution in feeds for cod and sea bass (Hansen *et al.*, 2006, Refstie *et al.*, 2006, Olsen *et al.*, 2007, Bonaldo *et al.*, 2008). A similar lack of severe enteritis-like changes has also been noted in other species of marine fish, including Egyptian sole (*Solea aegyptiaca*), cobia (*Rachycentron canadum*) and Atlantic halibut (*Hippoglossus hippoglossus*) (Grisdale-Helland *et al.*, 2002, Bonaldo *et al.*, 2006, Romarheim *et al.*, 2008b).

It should be noted however that wheat gluten demonstrates none of the enteritic effects seen in salmonids fed with soybean meal (Storebakken *et al.*, 2000), highlighting the importance of the soybean meal as the probable enteritis causing ingredient, and confirming the widely used term of soybean meal-induced enteritis (Baeverfjord and Krogdahl, 1996). However more studies are still required on other plant proteins and using other commercially important aquaculture finfish species (Tacon, 1997, Drew *et al.*, 2007) to fully elucidate the implications of substituting fish meal on the health of farmed finfish.

1.1.6 Future protein: where do we go from here?

It is clear that aquaculture feeds are changing, and have been for some time. Alternative protein sources are becoming widespread due to the massive increase in fish meal prices and the reduction in relative availability. However, demand for such alternative sources is rising as human population grows, fuelling demand for the protein source itself (e.g. soybeans) and also for farmed fish. This increase in demand leads to an increase in price, in turn reducing the relative economic benefits of replacing fish meal. Indeed, this long term upwards price pressure has already begun to show, with prices of seeds and meals, rising on average 65% and 90% respectively between June 2005 and June 2009 (FAO, 2009). However, it is important to note that as of June 2009, soybean meal prices were $441 a tonne, still less than half the price of fish meal at $1164 a tonne (Barrientos, 2009). It is

therefore important to prevent the industry from becoming reliant on the alternative protein sources investigated in the last few years, but to ensure that new alternatives are continually being investigated in order to maintain the ecological and economical sustainability of aquaculture.

A protein source that is arousing debate within the industry at present is that of seaweed, with research having been carried out to date on *Ulva spp.*, *Porphyra spp.* and *Gracilaria spp.* as protein sources for finfish. *D. labrax* have been shown to grow successfully on two species of dried and powdered seaweed (*Ulva rigida* and *Gracilaria bursa-pastoris*) at 10% inclusion, but only at 5% inclusion on a third species, *Gracilaria cornea* (Valente *et al.*, 2006). Higher inclusion levels were not tested. In the case of *G. morhua*, a 30% fish meal replacement (16.5% inclusion), by *Porphyra spp.* had no effect on growth and shows promise (Walker *et al.*, 2009). Despite the moderately promising results from these early studies, a precautionary approach has been recommended by researchers concerned about the risk of disease transmission by feeding protein produced in the marine environment (di Pietro, 2009). Costs associated with the labour intensive production of marine macroalgae may also limit its usefulness in replacing fish meal (di Pietro, 2009) although the economic costs or benefits at present are not clear and require more concentrated analysis (Valente *et al.*, 2006, Walker *et al.*, 2009).

A further ingredient of interest is protein produced by and/or extracted from yeast. Two studies by Lunger *et al.* (2006, 2007) have investigated a commercially produced organically certified yeast protein, NuPro® in feeds for *R. canadum*. They demonstrated that such a protein source can provide 25% of dietary protein (Lunger *et al.*, 2006) while addition of methionine, tryptophan, and taurine can allow this level to be increased significantly (Lunger *et al.*, 2007). Craig and McLean (2005) also demonstrated that 25% of dietary protein in the same species can be provided by NuPro®, and that no significant effect was noted until 75% protein substitution. In *O. niloticus*, this was seen up to 100% protein replacement. In terms of other yeast sources, little is known about protein rich marine yeasts. But with many containing more than 30% protein in terms of cell dry weight, essential amino acids (especially lysine and leucine) and enzymes which can transform the waste products such as starch, protein, cellulose, and xylan into cell protein (Chi *et al.*, 2009) the potential

appears to be there for further research. Again, price information is scare, but potential is high for this alternative protein source.

By-product or processing waste meals are proteins which require no new production in terms of farming and so pose no land use conflicts, which can occur in the production of seeds and pulses. Such meals usually originate from the processing of animals protein for human consumption, such as meat, bone, poultry and fish. Wang *et al.* (2006) investigated poultry by-product meal, meat and bone meal and feather meal in feeds for cuneate drum (*Nibea miichthioides*). They found that the first two of these protein sources could be used at 17% (to replace 50% of the fish meal) and 10% (to replace 30% of the fish meal) respectively, while feather meal resulted in lower SGR and whole faba beans compared to those of fish fed the control feed. This may have been due to low protein digestibility, as recorded by Tibbetts *et al.* (2006) at 62.4%. In support of the potential of such meals, Ai *et al.* (2006) showed that 45% of fish meal could be replaced by meat and bone meal in diets of yellow croaker (*Johnius dussumeri*) without significantly reducing growth. These inclusion levels are further increased by lysine and methionine supplementation (which consequently increases cost) with poultry by-product meal and meat and bone meal replacing 66.7% of fish meal protein in feeds for gibel carp (*Carassius auratus gibelio*) (Hu *et al.*, 2008). The available costs of these meals as of July 2009 was $540 (feather meal), $401 (meat and bone meal) (Steevens and Sexten, 2009). The price of poultry by-product meal was not available, but Pine *et al.* (2008) reported that despite complete replacement being feasible for sunshine bass (*Morone chrysops* X *M. saxatlis*), reduced revenue over feed costs would render it an undesirable protein source.

The last of the potential protein sources listed as being produced as a result of protein production for human consumption is that from the fishing industry. These meals can either be produced during the processing of seafood, such as shrimp processing waste, or by the rendering of by catch or incidental non-targeted species taken while fishing for targeted species, which in itself would offer an additional income source to fishermen as well as making use of normally discarded fish and shellfish. *O. mykiss* fed diets containing shrimp processing waste and Pacific whiting meal had significantly lower weight gain and higher feed conversion ratios than the control

feed, whereas shrimp by catch meal (various fish species) was comparable to the control (Hardy *et al.*, 2005). The authors suggested that palatability was the cause of the poor performance of Pacific whiting meal, while shrimp processing meal was significantly lower in protein than the other meals at only 54%. Fish silage, a liquid produced from chemically or enzymatically digested fish carcasses or waste, has also been shown by several authors to represent a potential protein source in feeds for farmed fish (De Arruda *et al.*, 2007). Although exact price information is unavailable for such fisheries by-products, it might be expected that they could be a potentially cost effective, readily available and ecologically sustainable protein sources (De Arruda *et al.*, 2007).

It should be noted that this selection of protein sources is by no means exhaustive. It is aimed to highlight the range of sources that are available over and above the current crop of plant proteins, and the fact that not all of them will be suitable for use in aquaculture feeds. However, it does show that there are many protein sources which have not been as extensively trialled as have some plant proteins, in particular soybean, which are cheaper than fish meal, and pose less of a risk of conflict with human consumption needs and result in fewer intestinal problems as is seen with some plant proteins. It is important that such factors are considered when identifying future proteins for use in formulated aquaculture feeds.

1.2 Effluent management in aquaculture

Suspended solids produced from aquaculture are mainly made up of uneaten feeds, digesta and micro-organisms (Viadero and Noblet, 2002, Tacon and Forster, 2003). The removal of these solid wastes from fish farm effluent or the reduction of such wastes released from cage farming operations is central in the effort to reduce the particulate biological load of effluents and in reducing potential impacts on stock safety and on the environment. Estimates of total suspended solids (TSS) from aquaculture in the form of both feeds and faeces range from 29 to 78% of the total input of carbon, measured under sea cages (Pearson and Black, 2001), with deposition under cages exceeding the background sedimentation measured 1 km away by between 8 and 25 times (Holmer *et al.*, 2007). TSS in cold water single pass systems, serial reuse systems, partial reuse and recirculating aquaculture systems

(RAS) has been shown to be 1.3, 5.7, 48 and 152 mg l^{-1} respectively (Chen *et al.*, 1997). In the untreated effluent from flow-though farms, Cripps and Bergheim (2000) showed TSS to be around 5 to 50 mg l^{-1}, commonly carrying 7 to 32% of the total nitrogen and 30 to 84% of the total phosphorus in the wastewater. In RAS, TSS has also been shown to be relatively low, at between 9 to 11 mg l^{-1} (Johnson and Chen, 2006, Pfeiffer *et al.*, 2008), apparently in contrast to the findings above. It should be noted therefore that estimates and measurements vary widely between different operations, even those utilising the culture system, depending on level of intensity, species, feeds, and effluent processing.

There appears to be a relatively wide range of estimates for the amount of total phosphorus which is present from aquaculture effluents in the particulate form, compared to the dissolved fraction. Kristiansen and Cripps (1996) reported that 47 to 84% of total phosphorous (and 25% of total nitrogen) was bound into particulate matter. Within this wide range fall the values found by Holby and Hall (1991) and Piedrahita (2003) who reported of the phosphorus load to the environment from a marine fish farm, 60 to 66% was in the particulate form. Chen *et al.* (1997) state that total phosphorous is mostly in the dissolved form, with only 33% in the particulate form, while Lemarie *et al.* (1998) reported that 56% of total phosphorous on average is in the particulate form, reducing to 28% where vigorous agitation of the water column takes place. Phillips *et al.* (1993) in Garcia-Ruiz and Hall (1996) found that 40% of total phosphorous in faecal material was lost from the settling solids as particles settled within the first 5 hr post production while Piedrahita (2003) reports that 10% of total phosphorous leached out of faeces settling in 30 m of water.

These data would appear to highlight the need for time frame and water conditions to be taken into account when investigating the form in which nutrient elements, in particular phosphorus, are found in the water column or sediment. However it does show that a large proportion of the total phosphorous load can be removed relatively easily from the effluent water, and as dissolved nutrients cannot be removed through traditional particle separation techniques (Cripps and Bergheim, 2000), this is of great importance. Indeed Piedrahita (2003) noted that a high proportion of the total phosphorous load remained in a screen filter backwash and therefore rapid solid removal is effective in capturing and separating substantial portions of the total

phosphorous produced in the effluent from aquaculture operations. This knowledge would also allow planners to ensure the effective siting of installations such as bivalve beds in the case of integrated multi tropic aquaculture operations could maximise the removal of total phosphorous and other nutrients.

The sizes of fish derived faecal particles in water range over four orders of magnitude, from 1 μm to approximately 2000 μm (Bagnold and Barndorffnielsen, 1980). Biological, chemical and physical factors acting upon faecal particles suspended in an aqueous medium can affect the sizes of those particles with time (Hargrave, 2003). Brownian motion and low intensity turbulence can result in collisions between particles and can encourage flocculation, whereas higher intensity turbulence due to water currents, pumping or fish movement can lead to the shearing apart of flocs. Naturally large particles such as grits and undigested food particles will tend to sediment easily as settling velocity depends in part on particle size (Pillay, 2004), and they will therefore not affect the wider environment around sea cages and are relatively easy to remove from the water in land based systems. As many of the chemical reactions and microbial growth occur on the surface of the particles, particle surface area, volume and number are also important factors to consider (Patterson *et al.*, 1999).

The potential effects of TSS produced by aquaculture are wide ranging. In RAS, concentrated sediments can cause clogging of the screens, filters and the biofilter, cause secondary ammonia production increasing biofilter loading and increasing biochemical oxygen demand, provide host sites for pathogens to develop and cause direct physical damage of the fish (Patterson *et al.*, 1999, Viadero and Noblet, 2002). Several technologies have been employed and continue to be refined to ensure that as much of the TSS is removed prior to recirculation. Direct discharge of dilute effluents to the environment in the case of sea cage farming or semi intensive land based or flow through farms can result in eutrophication of waterways and inshore sites, overwhelming of the feeding capacity of benthic organisms, the formation of bacterial mats and anoxic conditions, and silting of coastal areas and riverine systems (Silvert, 1992, DEFRA, 2006, OSPAR, 2006).

1.2.1 Physical properties of effluents

Recently, researchers have also become aware of the need to determine the physical characteristics of effluents, however information pertaining to this is highly variable in the published scientific literature (Magill *et al.*, 2006) and the potential of novel ingredients to alter these characteristics is largely missing (Reid *et al.*, 2009). Environmental impact from flow through fish farms due to feed and faecal waste can be substantial, and the impact of such wastes on mechanical effluent treatment efficiency in RAS is also of importance. The removal of solid wastes from fish farm effluent is important in reducing the overall particulate biological load of the effluent and subsequently in reducing the impact of the operation on the environment. The removal of this waste is carried out by settlement in basins and separators (Veerapen *et al.*, 2005) or by physical filtration of the raw effluent through membranes (Viadero and Noblet, 2002), foam fractionation (Cripps and Bergheim, 2000, Brambilla *et al.*, 2008), microsieves and sand filters (Kristiansen and Cripps, 1996). It is clear that waste particles must be larger than the pore size of a screen or filter to be removed or they must be large enough to settle within the retention time of the water to enable effective sedimentation (Brinker *et al.*, 2005a).

If a proportion of the waste particles are smaller than the critical sizes for filtration or settlement, then that proportion of the particulate biological load will remain in suspension and will be discharged to the environment (in the case of flow through farms) or enter the biological filtration unit (in the case of RAS). The former has obvious direct environmental effects such as eutrophication of freshwater bodies and/or benthic smothering of sub littoral and deepwater marine environments. The latter can result in water quality issues within a RAS through stressing of the biological filter microflora, rendering it unable to process the levels of nitrogenous waste presented to it, resulting in a build-up of toxic substances such as ammonium and nitrite in the circulating water.

In open water cage systems, the particle size and settling characteristics will affect the radius of impact of the farm. Larger particles would tend to settle in short amount of time resulting in a high local benthic impact, while fine particles would result in wider ranging effects of lower intensity. Therefore the stability of waste suspensions, the likelihood of particles to fall out of suspension, the filtration efficiency and the

flow properties of aquaculture effluent all have an important part to play in planning, managing and engineering such systems.

1.2.1.1 ***Particle size distributions.*** As noted previously, the range of particle sizes in suspended fish faeces is wide; however there is some consistency among published studies in the positions of peaks within faecal particle size distributions. Brinker *et al.* (2005a) observed peaks for faeces stripped from *O. mykiss* at 32 to 51, 128 to 323, approximately 400 and above 600 μm. At a lower particle size resolution (i.e. with larger particle size range intervals), peaks were also noted at 23 to 55 and 105 to 500 μm in the raw effluent from a RAS for *O. niloticus* (Pfeiffer *et al.*, 2008). Maillard (2005) reported three peaks occurring at 1.5 to 30, 105 to 210 and above 210 μm in effluent from *O. mykiss* farms. When the median values of the peaks derived from the combined data from these three studies are taken, three composite peaks appear at 16 to 42, 158 to 303 and above 400 μm. It is interesting to note that a similar pattern of particle size peaks is also seen in municipal wastewater effluents. Zhang *et al.* (2007a) recorded peaks at approximately 20, 100 and 300 μm, while Schubert and Gunthert (2001) found peaks at between 6 and 30 μm, 102 to 222 μm and 438 to 510 μm near trickling filters prior to settlement.

It would be unclear without microscopic examination whether particles at any given size range are single particles or flocs. Indeed, the formation of flocs in many cases may be encouraged, as an effluent containing coarser particles would have a greater removal potential by filtering or settlement. However, in order for flocculation to occur, particles must first be able to come into close proximity under the influence of Brownian motion or water currents so as to be able to collide, allowing inter-particular forces such as van der Waals to allow them 'stick' together. The potential proximity of any two particles, and therefore the likelihood of those particles to collide, is largely dependent on the charge on the surface of the particle.

1.2.1.2 ***Zeta potential.*** The arrangement of charge on the surface of a particle (the dispersed phase) and the charges of counter-ions (oppositely charged) and co-ions (like charged) in the surrounding liquid (the continuous phase) is organised into an electrical double-layer (Fig. 1). This consists of the Stern (inner) layer and the diffuse (outer) layer (Hunter, 1988, Shaw, 1992). The distribution of charge can be

quantified in the laboratory through the measurement of the zeta potential (ζ) of the particles, which refers to the electrostatic potential at the surface of shear between the charged surface and the electrolyte solution (Shaw, 1992).

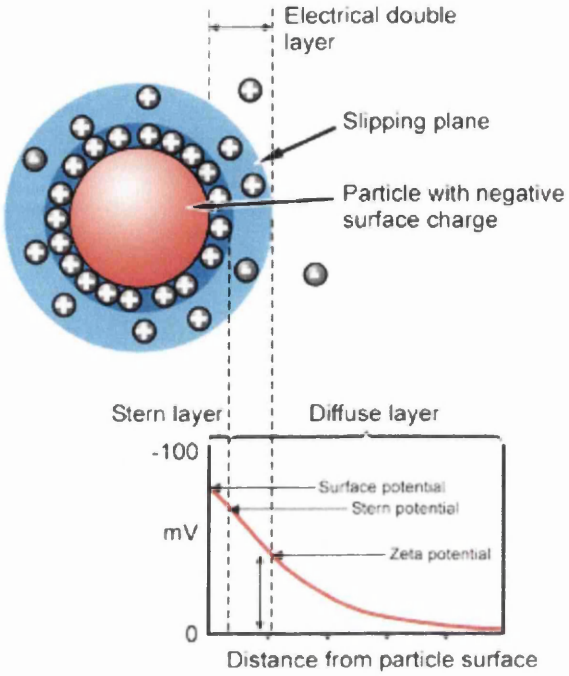

Figure 1: Diagrammatic representation of the arrangement of charge of the surface of a faecal particle, where the positive charge on the surface of the particle (the dispersed phase) and the negative charge of the counter-ions and positive co-ions in the surrounding liquid (the continuous phase) form the electrical double layer. Also shown is the surface of shear at which point the zeta potential is measured. Reproduced from Malvern Instruments Ltd information document (2009).

Zeta potential is an indirect measurement of the charge on particles, its value determining the extent of the electrostatic forces of repulsion between charged particles, in turn indicating the extent of stability of the suspension. A high positive or high negative ζ will therefore increase the electrostatic repulsion between particles, reducing the likelihood of collision. Through the analysis of ζ one can determine the isoelectric point (IEP) of the suspended particles. The IEP is defined as the point at which the net charge, and therefore electrophoretic mobility (Uε - the velocity of a particle per unit electrical field (Hunter, 1988)) is zero (Shaw, 1992), and is the point at which the likelihood of collision and flocculation is at its highest. This is because the diffuse outer layer is completely dispersed, there being zero charge to attract free counter ions in solution to the surface of the particle. This in turn allows the particles to come into close proximity to one another and collide.

The ζ of particles is dependant both on the surface properties of the particle and on the chemical properties of the continuous phase; in the current study this is the water in which the fish are cultured. The salinity of the water can affect the extent of the double layer and can have some affect on the stability of a system (but not the IEP) by affecting the potential proximity of particles to one another. pH can have a highly significant effect on the ζ and can alter the IEP, and therefore can have a pronounced effect on the stability of the faecal material and the potential of the aquaculture system to remove particulate waste effectively. These forces of repulsion can also change with the addition of a flocculant, and as such the ζ serves as a destabilisation indicator (Harif and Adin, 2007) and can be calculated if the Uε of the sample is known.

Plots of ζ at different environmental conditions, usually pH, often show a distinct shape depending on the surface properties of the particles and the chemical properties of the continuous phase. It might be expected that the amino acids would be the most important determinator of surface chemistry, as protein is generally the greatest single ingredient in a feed. In this respect, the ζ should be determined by the charge characteristics of carboxyl (–COOH) groups, imparting a negative charge, and amino ($-NH_2$) groups, imparting a positive charge. The value for the acidity constant (K_a) and its negative logarithm (pK_a) of the functional groups suspected of being responsible for determining the IEP can help in proving or otherwise the likelihood of the isoelectric point being attributable to a particular functional group. The acidity (dissociation) constant is a measure of the proton donating strength of an acid, and is the point at which the protonation of a proton acceptor balances proton dissociation from the acid within the solution (Atkins and Jones, 2002), and thus determines the IEP. Paired to this is the pK_b, the basicity constant which is the association constant for protonation of the base to form the conjugate acid.

The values of the pK_a and pK_b and their close affiliation with the IEP of particulate or membrane systems has also been shown in studies in which both have been investigated. It is known that the average pK_a and pK_b of a range of common amino acids are 2.17 and 9.66 respectively. Cai *et al.* (2006) investigated titanium thin films' surface chemistry in relation to ζ and showed that Ti-COOH had the lowest IEP (about pH 3.3) while Ti-NH_2 had IEP of about pH 7. Valle-Delgado *et al.* (2003)

showed that the IEP of amphoteric latex with surface functional groups of –COOH and $–NH_2$ only was approximately pH 4 in a saline electrolyte. Amphoteric Polyamide 6 foil (used in food packaging) was found to have an IEP of pH 4.2, which increased to pH 8.1 after treatment with NH_3 plasma, which served to introduce more surface amino groups (Tusek *et al.*, 2001). These results demonstrate the fact that –COOH and $–NH_2$ groups do affect surface properties significantly, and that the IEP and therefore ζ can be significantly affected by them.

The shape of a ζ plot will be highly dependent on the properties of the surface being tested, and it would therefore be expected that the –COOH and $–NH_2$ groups would demonstrate a certain plot shape. Jean *et al.* (2006) noted a rapid drop phase occurring between pH 3.0 and 6.0 preceded and followed by a flattening out of the curve when analysing serum heat-induced milk protein aggregates. Tusek *et al.* (2001) analysed amphoteric Polyamide 6 foil and observed two plateaus either end of a rapid drop between pH 3.5 and 6.5, and noted that this was due to the amphoteric nature of their study material. A study on waste water treatment plant effluents also recorded a rapid drop up to pH 6, followed by a slight plateau and a further rapid drop up to pH 8 (Schrader *et al.*, 2005). Aguedo *et al.* (2005) showed a marked drop in ζ between pH 2.0 and 4.0 of *Yarrowia lipolytica* cells, with a plateau forming from pH 4.0 to 9.0. A similar study on the same species of yeast demonstrated a plateau between pH 3.0 and 9.0 with an increase in gradient above and below these values (Amaral *et al.*, 2006). It would therefore be expected that if indeed the surfaces of the faeces particles produced as part of the subsequent experiments were dominated by amino acids, a curve with certain distinct shape characteristics would be formed. It might be expected that rapid drop phases would be noted at low pH (acid) and high pH (basic) conditions, perhaps preceded and followed by plateaus.

1.2.1.3 ***Settling characteristics.*** Settling velocity information for fish faecal material taken from the literature is presented in Table 1. It can be seen for this information that a high variation is seen in settling velocity, with velocities for all species ranging from 0.48 to 6 cm s^{-1}, and with *S. salar* ranging within that from 1.5 to 6 cm s^{-1}, *D. labrax* at 0.64 to 0.70 cm s^{-1} and *G. morhua* at 3.7 cm s^{-1}. These settling velocities

Table 1: List of studies which have determined the settling rate of faecal material voided from finfish aquaculture species, adapted from Magill *et al.* (2006) and Reid *et al.* (2009). ND = no data available.

Author(s), (year)	Species	Collection method	Particle settling velocity ($cm\ s^{-1}$)	Size range measured	Number of particles	Notes
Iikura (1974)	*Seriola quinqeradiata*	ND	5.0	ND	ND	
Gowen and Badbury (1987)	*Salmo salar*	ND	1.7 – 6.0	ND	ND	
Findlay and Watling (1994)	*Salmo salar*	In situ trap	2.0	ND	ND	Measurements over 10 cm distance
Panchang *et al.* (1997)	*Salmo salar*	Siphoning	3.2	ND	50	70% of observations 2-4 $cm\ s^{-1}$
Elberizon and Kelly (1998)	*Salmo salar*	From tank outlet	2.9 1.5	> 2 mm > 0.5 mm	90 90	Approximate fish weight=25 g; in freshwater
Chen *et al.* (1999)	*Salmo salar*	Stripping Netting	5.4 5.3	4.0 mm 6.8 mm	9 24	Mean fish weights =1 kg and 0.75 kg; in seawater
Wong and Piedrahita (2000)	*Oncorhynchus. mykiss*	Stripping and from raceway	1.7	ND	ND	Median (mass based) settling velocity
Cromey *et al.* (2002)	*Salmo salar*	In situ trap	3.2	ND	44	Mean fish weight 3.39 kg
Chen *et al.* (2003)	*Salmo salar*	Netting	5.8	ND	ND	
Malačič and Forte (2003)	*Dicentrarchus labrax*	Polythene trap	0.64	ND	ND	Particle ensemble measured
Ogunkoya *et al.* (2006)	*Oncorhynchus mykiss*	Siphoning	2.7 – 3.9	ND	ND	
Magill *et al.* (2006)	*Dicentrarchus labrax* and *Sparus aurata*	In situ trap	0.70 0.48	0.3-6.2 mm 0.3-2.5 mm	1021 1042	Settling velocity and volume distributions measured
Cromey (2009)	*Gadus morhua*	Stripping	3.7	ND	ND	

will also depend in some part on feed type, length of exposure to water, particle size, and faecal collection method, among others things. As such it is difficult to suggest a definitive value for any particular fish species or particle size range.

In developing DEPOMOD, and model for predicting aquaculture waste deposition from cage farming operations (discussed in more detail later), Cromey *et al.* (2002) found that using a mean settling velocity assigned to all model faecal particles resulted in an inaccurate representation of the observed data. From this information and the wide range of measured settling velocities, it is clear that more work is necessary on this aspect of faecal physical analysis to ensure that results are verified and can be relied upon when used as is or entered into models of particulate deposition.

1.2.1.4 ***Faecal stability (rheology).*** Rheology is the study of the flow and deformation of fluids, and an important aspect of the discipline incorporates the study of the viscosity of fluids. The viscosity or physical consistency of faecal wastes can have an impact on the stability or cohesiveness of the faecal material, in turn affecting the physical removal potential or dispersion characteristics of the effluent, and all treatment, utilisation and disposal operations (Spinosa and Wichmann, 2004). This is due to the stability of an effluent having implications on its ability to resist floc breakage and disaggregation and its likelihood to disperse (Reid *et al.*, 2009).

Brinker *et al.* (2005b) reported that both alginate and guar gum, common dietary binders, were capable of increasing the viscosity and effluent removal potential in a dose-dependent manner in feeds for *O. mykiss* in a flow through farm. Leenhouwers *et al.* (2004) investigated the addition of guar gum in feeds for *D. labrax* also finding that that digesta viscosity was higher in feeds containing the guar gum binder. An increase in digesta viscosity at high dietary levels of starch was also linked to higher faeces removal in *O. niloticus* (Amirkolaie *et al.*, 2006). The rheology of effluents can furthermore have significant implications in effluent pumping and pipeline transport (Sanin, 2002) in closed recirculation systems. It is therefore necessary in closed aquaculture systems to achieve a balance between feed properties which encourage a high faecal removal potential (high viscosity/large particle sizes), but

which also encourage low cost and trouble free pumping and pipeline transport (low viscosities).

Ogunkoya *et al.* (2006) investigated the effects of feeding soybean meal with an enzyme cocktail on waste outputs and growth in *O. mykiss*. They determined that waste output and growth was not affected, but cohesiveness was reduced by enzyme addition and soybean meal inclusion, with the latter also affecting sinking speed. The authors discussed the possibilities that the observed properties could reduce localised deposition and lessen the impacts on local benthic biota in sea cage farms (Reid *et al.*, 2009) but could reduce solid waste recovery on land-based fish culture operations.

The effects of feed additives designed to alter the physical characteristics of effluents on the health of farmed fish must also be taken into account if their use at relatively high levels in feeds is to be seriously considered in the industry. In general, the apparent digestibility coefficient of dry matter, protein, ash and energy decreases with increasing inclusion levels of guar gum, in African catfish (*Clarias gariepinus*) (Leenhouwers *et al.*, 2006). A similar reduction in the apparent digestibility coefficient of dry matter, organic matter, fat, protein and starch was found in *O. niloticus* compared with feeds without guar gum (Fagbenro and Jauncey, 1995, Amirkolaie *et al.*, 2005). These effects may be explained by the increase in digesta viscosity induced by guar gum (Amirkolaie *et al.*, 2005, Leenhouwers *et al.*, 2006), which is known to gel during mixing prior to pelleting and which may bind the nutrients (Fagbenro and Jauncey, 1995) and limit diffusion of digestive enzymes (Choct *et al.*, 1995). Refstie *et al.* (1999) reported similar results to those seen with guar gum when using low protein soya products to determine the effects of non-starch polysaccharides in feeds for *S. salar* with a negative effect on the digestive processes. However in this case no effect of feed on the viscosity of the intestinal contents from salmon was noted and the exact reason for the reduced digestibility was not determined by the authors, but may have been related to digestive tract alterations as discussed previously. In agreement with these findings, Forde-Skjaervik *et al.* (2006) found that 24% inclusion (crude protein) of soy did not influence the viscosity of cod digesta, and viscosity differences could therefore not explain observed differences in digestibility between the feeds.

In contrast to those results described above, Brinker *et al.* (2005b) reported that there were no significant negative side effects on the health of *O. mykiss* or on the digestibility of macronutrients when using guar gum as a binder, despite a significant increase in digesta viscosity. Leenhouwers *et al.* (2004) drew the same conclusions when studying feeds for D. *labrax*. A study using *O. nilotica* found a significant positive correlation between stomach chyme viscosity and protein apparent digestibility coefficient (Amirkolaie *et al.*, 2006), a complete reversal of those results outlined previously. In this case, it was suggested that an increased viscosity in the stomach may have increased the retention time and thereby prolonged the time of exposure of the digestive enzymes and low pH in the stomach. In summary, it appears that the addition of binders does increase affect the viscosity and stability of faeces. However, it is difficult to conclude whether increasing the viscosity of the chyme has any effects on the digestibility of the feeds, and this may indeed be species specific.

1.2.2 Impacts in open water culture systems

The behaviour and residence times of waste particles in the water column (Sutherland *et al.*, 2001) and on the sea bed (Milligan and Loring, 1997) depends on the hydrography, topography and geography of the receiving water body and seabed, the tidal flow and currents, residual circulation, patterns of turbulence, and wind and wave energy, which determine the large scale patterns of dispersion (Holby and Hall, 1991, Fernandes *et al.*, 2001, Hargrave, 2003). The effects of suspended solids released from open water fish farms, for example sea cages, therefore depend in a large part upon the siting of the farm (Holby and Hall, 1991).

The differences noted between inshore and offshore operations exemplify the effects of siting, as waste loadings at offshore farms can be much lower than inshore sites due to deeper water and the possibility of cages moving on their moorings, thereby spreading the wastes over a larger area (Read and Fernandes, 2003, DEFRA, 2006). This area may be very much larger than the cage area itself, as demonstrated by Holby and Hall (1991) at an *O. mykiss*, cage farm in the Gullmar Fjord in Sweden, with a bottom depth of 18 to 21 m below the farm. The authors report that farm sediment was observed in an area of 3.8 times the area of the farm itself. In comparison, it would be expected that offshore farms would operate in > 100 m

water depth. Phillips *et al.* (1993), in Garcia-Ruiz and Hall (1996), and Cromey *et al.* (2002) reported that the sedimenting velocity of fish faeces was between 3.2 cm s^{-1} and 4.0 cm s^{-1} which equates to more than 42 min in the water column (assuming a straight path to the sea bed) in the open water example as opposed to approximately 10 and 8 min in the fjordic example. This shows the scale of the difference in terms of time available for prevailing conditions to affect the trajectory and the size of the sinking particle between inshore and offshore sites. It therefore appears that siting farms in deeper water could reduce the severity of effects on the benthos, but may cause low intensity effects over a wider area. Indeed, a review of methods to collect particulate materials from moored cages by Pillay (2004) resulted in a conclusion that careful consideration of the siting of cages is the best way to minimise the impact of sediments and to disperse solids.

The amount of particulate organic matter discharged to coastal areas in Europe has increased as the aquaculture industry has expanded over the last ten to fifteen years (MEMG, 2004), mainly due to the increase in cage farm numbers around the shores of EU nations. However, statutory controls put in place with this expansion in mind have ensured that any significant impacts are restricted to the immediate vicinity of sea cages. Such statutory controls are based on a system whereby predictive modelling is employed prior to granting licenses for cage farm sites followed by seabed monitoring. The results are environmental impacts which may be significant locally, but insignificant in the wider context; an example being Scottish sea lochs where the area of the seabed that is impacted is often very small, and rarely extends to beyond 50 metres from the perimeter of the cage (MEMG, 2004). In support of this, a study at a farm in Tasmania indicated that benthic communities under and 10 m from a sea cage showed signs of moderate disturbance with semi to highly enriched conditions, whereas beyond 30 m there was no disturbance, with normal sediments (Ye *et al.*, 1991).

Modelling of waste discharges into open water sites can help to provide information on environmental issues to regulatory agencies, to aid in the design of monitoring strategies, to provide advice on coastal zone management and provide advice on site location (Silvert and Cromey, 2001). Such models developed for this purpose are the general finfish Aquaculture Waste Transport Simulator (AWATS) (Dudley *et al.*,

2000), DEPOMOD for salmon farms (Cromey *et al.*, 2002), and CODMOD for cod farms (Cromey *et al.*, 2009). Accurate predictions of the potential impacts on the benthos require calculations of the flux of particulate materials, which can be determined in the laboratory, knowledge of sea bed dynamics and the identification of processes associated with the degradation of materials once settled. With the dynamism of the marine or fjordic environment, models can only give an approximate footprint of effects from a cage farm. But the errors due to environmental dynamism are relatively minor compared to those due to generalisations made about faecal production, and therefore the problem of the particle flux is never fully addressed. This is because different types of particles with different shapes, densities, and charge distributions are involved and these properties can alter as they settle. Flocculation or aggregation of fine particles can also play a major role in altering the sedimentary characteristics of particulate wastes by altering settling velocity of the suspended matter (Milligan and Loring, 1997). The flocculation rate is primarily dependant on concentration and adhesion efficiency, and so small changes in the balance will directly affect settling velocity and therefore particle dispersion (Hargrave, 2003).

1.2.3 Impacts in closed or partially closed aquaculture systems

Particulates can be considered pollutants when they become suspended in RAS water (Lee and Lawrence, 2001). It is therefore very important that they be removed a soon as possible following production, and control of TSS must be the first priority in order to reduce the impact of the material (Chen *et al.*, 1997). The stages of solids management in aquaculture systems comprise feed quality manipulation, feeding management, pre-treatment, primary separation, secondary thickening, sludge stabilisation and its reuse or disposal (Cripps and Bergheim, 2000).

Widely used particulate removal methods involve using microsieves, rotary drums, or rotary disc screens, which usually remove particles over 60 μm (Kelly *et al.*, 1997) with efficiencies between 65% and 97% (Viadero and Noblet, 2002, Borges *et al.*, 2003). By adding membrane separation as a treatment step, particles with diameters down to 0.05 μm can be removed (Viadero and Noblet, 2002). Membrane separation however has a variable performance as it is highly dependent on many parameters such as the physical, chemical and operating conditions. Also, removal of

particles below 50 μm is usually not economically viable (Patterson *et al.*, 1999) and therefore membrane filtration is generally more useful and viable as a secondary filtration or 'polishing step' in the water treatment, further reducing biological oxygen demand and the load on the biofilter (Viadero and Noblet, 2002). Foam fractionation can also be used a fine particle removal system, having been shown to be especially effective at removing particles > 60 and < 1.2 μm from RAS water (Brambilla *et al.*, 2008), while wastewater from foam fractionators have also been shown to contain particles < 30 μm (Chen *et al.*, 1993a).

The inefficiencies in removing very small particles are shown in studies investigating particle sizes in culture water. In a recirculating cold water system *S. salar*, Patterson and Watts (2003) reported that the vast majority of particles were less than 10 μm and mostly between 2 μm and 6 μm, while Chen *et al.* (1993) in Piedrahita (2003) found that 48% solids mass in the culture water of a recirculating system was particles below 10 μm. However particles up to 20 – 25 μm are not consistently removed and can accumulate and deteriorate water quality (Viadero and Noblet, 2002) in cases where a foam fractionation process is absent. It is unlikely that shearing of larger particles, through the action of disturbances such as stock activity, aeration and stock capture (Pillay, 2004), plays a significant role in producing small particles. This is because the design of tanks in modern RAS, incorporating systems such as swirl separators, encourages waste to settle quickly (Pillay, 2004). It has been found that including such processes in aquaculture operations can reduce TSS by 82 to 85% (Summerfelt *et al.*, 2004, Johnson and Chen, 2006, Pfeiffer *et al.*, 2008).

Increasing the concentration of TSS prior to treatment would be the most desirable way of increasing filtration efficiency, an effect described by Cripps and Bergheim (2000), thereby reducing the overall TSS load in the culture water. Alternatively, altering feeds to produce intact high density faecal pellets which are easily removed from the culture water effluent stream would be a cost effective way for the end user to reduce TSS (Hardy, 2000, Piedrahita, 2003).

Partial reuse systems are those that discard approximately 20% of the system water with the effluent, returning the remainder to the culture tanks. Summerfelt *et al.* (2004) described a partial reuse system, and analysed the efficiency of solids removal

compared to other systems, in particular a serial reuse system whereby water flows from raceway to raceway and is discharged following treatment and is not reused. Side drains removed water from the culture tanks that was destined to be reused, containing 2.2 mg l^{-1} TSS, while a bottom drain removed solids at a concentration of 17.1 mg l^{-1}, 8.7 times greater than that exiting the side drain. The reused water was filtered through a 90 μm microscreen drum which removed 10 – 25% of total daily TSS, equating to 97% of the TSS in the system water. They found that 68 to 88% of daily TSS was removed through the bottom drain, which was then treated by a microscreen filter, with an average of 3.6 mg l-1 TSS being discharged following treatment. In total 82% of daily TSS was captured by the filtration system, compared to a capture efficiency of just 25 to 51% reported by Mudrak (1981) in Summerfelt *et al.* (2004) for the serial reuse system.

The removal of solids from systems in which filtering or screening is not used, i.e. those in which settling is the main form of suspended solid removal, relies heavily upon the density differences of particles (Patterson *et al.*, 2003). However, despite settlement being used traditionally in Europe and North America as the first stage of particle separation, the low residence times and high flow rates associated with more intensive land based farms makes this inefficient (Cripps and Bergheim, 2000). It is therefore more suited to the secondary de-watering or thickening of sludges prior to reuse or disposal.

The sludge which is produced from settling or filtering the TSS from the effluent water must then be treated in an environmentally and economically sustainable way. The treatment of the resultant sludge is therefore of the utmost importance to such operations. However the amount and form of nutrients released from culture systems depends upon the feed properties and the type of culture system, and therefore the composition of sludges can be highly variable (Cripps and Kelly, 1996) requiring uses for sludges to be determined on a case by case basis.

1.2.4 Effluent treatment

There are several methods for treating effluents produced from aquaculture (Fig. 2). The initial stage outlined here is the clarification or sedimentation usually involving ponds or tanks which allow matter to settle out of suspension, the design of which

would to some extent be dictated by the effluent characteristics such as the particle structure and settling properties (Pillay, 2004). However, as noted previously, sedimentation is not generally recommended as the only method for solids removal due to the poor performance in removing fines from the effluent water (Piedrahita, 2003), and thus the polishing steps are also included in this summary. Following clarification or sedimentation, the sludge is 3 to 6% TSS, and the overflow can water contain 10 to 30 mg l^{-1} TSS. Once sludge has been produced from the dilute effluent it is stabilised, which reduces the organic content of the sludge though complete oxidation, reducing the volume by 50 to 75%.

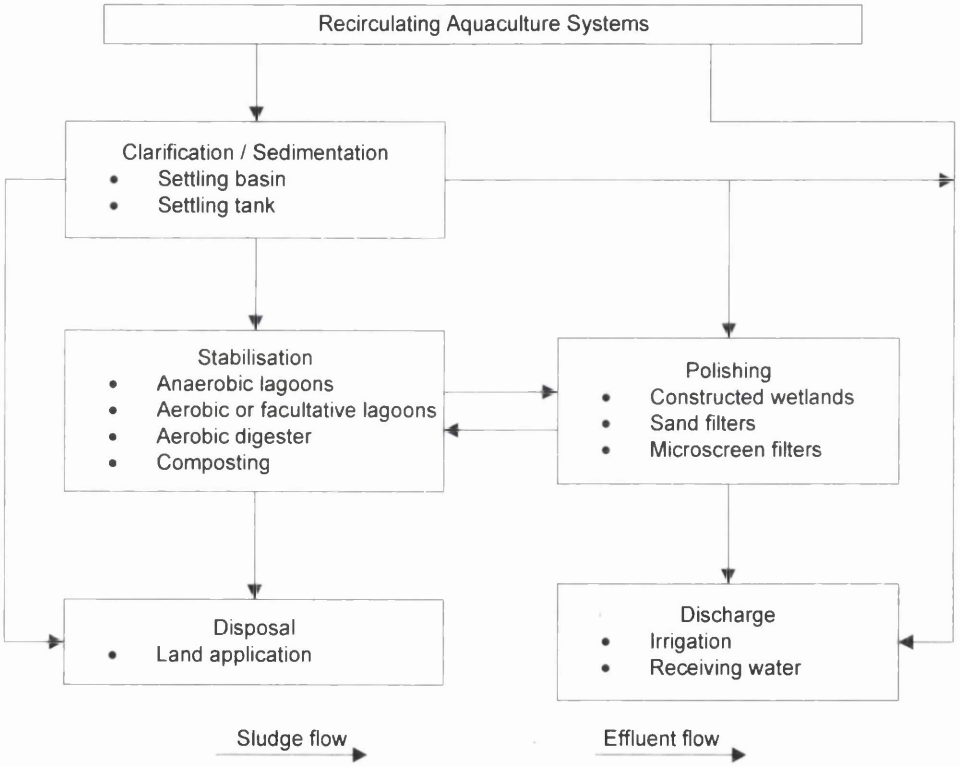

Figure 2: Options for aquacultural effluent and sludge treatment processes, adapted from Chen *et al.* (1997).

Sludge stabilisation can be carried out in several different ways:

- Anaerobic lagoon – can operate over a wide solids loading range with little maintenance, but is malodorous and therefore not suitable for use near population centres, are not very efficient at low temperatures and have a long sludge retention time.

- Aerated lagoon – this is a shallow pond that requires expensive aeration.
- Two stage lagoon – a lagoon with aerobic upper layers and an anaerobic lower layer, produces better quality effluent for irrigation, but there is little research in aquaculture use.
- Aerobic digester – produces a biologically stable sludge with low biological oxygen demand, is simple to use, suitable for use near population centres and has low set up costs, but a high cost of operation due to the need to aerate and a lack of a useful by-product.
- Anaerobic digester – produces useful methane and reduces pathogens in the sludge, but has a high initial set up cost, a long retention time, and is generally not recommended due to the large space requirements for the reactors.
- Composting – can reduce the volume of the sludge by 50 to 85% and produces an inert product, buts the high water content of aquaculture effluent (around 90%) makes it hard to compost and it must be dewatered to approximately 70% moisture prior to treatment.

Disposal of the sludge once produced can be as raw sludge following clarification or sedimentation stage, or in the stabilised form. The most appropriate option for use is as a fertiliser, with 3650 m^2 (0.90 acres or 0.365 hectares) of land required per 1000 kg fish d^{-1}, based on nitrogen requirements for crops and nitrogen content of sludges. However there are various problems associated with direct land application such as foul odours, the formation of a crust on the ground where the sludge is spread, the high cost of haulage and spreading, and a relatively slow nitrogen release. The latter is particularly a problem in areas that experience high rainfall due to high runoff potential and its use as a fertiliser in these areas is questionable. Therefore, for application in such areas, sludges should ideally be further stabilised and further reduced in volume. It should also be noted that in the case of land based marine operations, such as the culturing of marine fish or shrimp in ponds or RAS, sludges will contain very high levels of salt limiting their value as a fertiliser.

1.2.5 Managing the impact of aquaculture effluent

It is the quality, including feed physical characteristics and nutrient composition, and the quantity i.e. feeding rate and frequency, of feed that affects wastage and

excretion of particulates (Lee and Lawrence, 2001). Little can be done to decrease nutrient loads to the environment other than improve feed conversion ratios or reduce nitrogen and phosphorus concentrations in feeds (Boyd and Queiroz, 2001). In Europe a lot of attention has therefore been and continues to be given to limiting the inputs of nitrogen and phosphorus as feeds to marine cages in order to reduce the environmental nutrient loads. Since Denmark became the first nation to put in place legislation to limit the release of nutrients from aquaculture operations in April 1989, feeds for salmonids in particular have been transformed in the country and in Europe as a whole (Hardy, 2000).

The development of feeds and feeding practices within the industry is now concerned with the development and application of low pollution or 'environmentally friendly' feeds, the accurate management of feeds entering the system and the maximisation of utilisation of the nutrients to minimise nutrient loading (Lee and Lawrence, 2001). These low pollution feeds are being developed as high energy feeds, as in salmonid farming, where feeds with an increased fat content, reduced carbohydrate and protein content and an increased digestibility has significantly reduced waste production (Cripps and Bergheim, 2000).

In terms of improving the quality of water following filtration or sedimentation, manipulating the properties of the particles to encourage flocculation and the formation of larger particles can potentially lead to an increase in both the filtration efficiency and the sedimentation rate. The particle size and therefore the effluent removal and sedimentation potential can potentially be manipulated by engineering the feeds to alter the viscosity of the faeces, thereby increasing their stability, and encourage flocculation. A further possibility is manipulation of the ζ. Adding materials to the feeds that could act to bring the ζ closer to the IEP would result in a destabilisation effect, thus increasing particle size. Such ingredients could include charged ions or fibres, and the feasibility of this approach will be one of the subjects of the research herein.

1.3 Aims and objectives

The aims and objectives of this research are therefore to:

1. Determine whether a range of plant protein sources can replace fish meal in feeds for Atlantic cod and European sea bass, in terms of growth and performance;

2. Identify areas of analysis that can be used to characterise the physical properties of faeces produced as a result of offering experimental feeds to Atlantic cod and European sea bass;

3. Determine whether protein source has an effect upon these physical characteristics, to provide clearer information on the effects of fish meal substitution on effluent production and effluent management for farmed fish;

4. Investigate possible ways and means of reducing the impact of aquaculture effluent of the culture system, effluent removal, and the wider environment

Chapter 2

General materials and method development

All fish trials have been reported with reference to the "Guidelines for reporting the results of experiments on fish" (Brattelid and Smith, 2000).

2.1 Experimental rearing system

The experimental rearing system used in this project was based at the Centre for Sustainable Aquaculture Research (CSAR) in the School of the Environment and Society, Swansea University, a modern purpose-built facility consisting of a 750 m^2 controlled environment building, housing two industry grade RAS, plus a separate primary quarantine facility. Water and air temperature were centrally controlled, with all life support systems being electronically monitored and alarmed. Routine and specialist maintenance was carried out by the technical manager and staff at CSAR, to ensure the life support systems were operating optimally at all times.

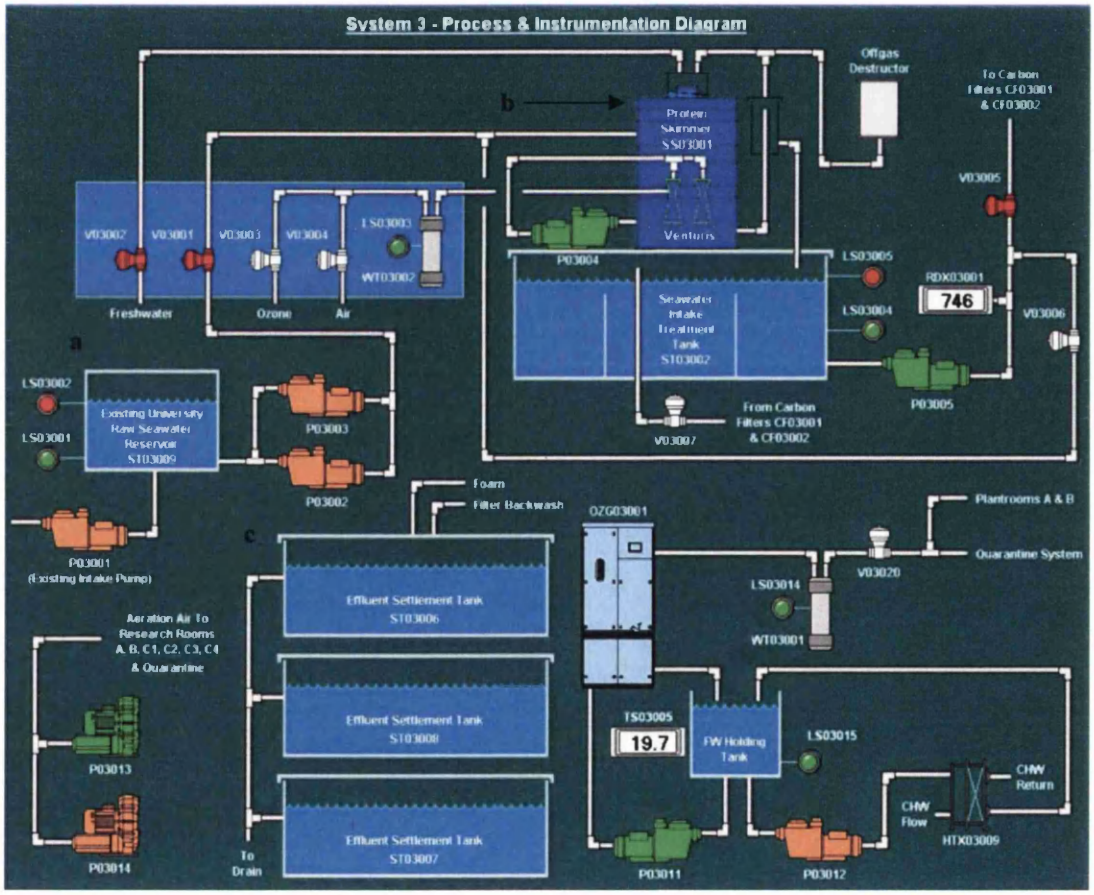

Figure 3: Screen shot of raw water intake and treatment system, showing 25 m^3 raw water holding tank (a), protein skimmer (b) and effluent settlement tanks (c).

Figure 3 illustrates the treatment process for incoming seawater. Raw seawater is drawn from Swansea Bay through sub-sand filters extending from the high water mark approximately 150 m seawards. The raw water is pumped to a 25 m^3 holding tank (a), from where it is pumped to an protein skimmer (b) which raises the redox of the water from approximately 200 mV to 750 mV and back down to 450 mV before sending it to a series of external temperature controlled storage tanks ready to be pumped directly into the RAS. While in the storage tanks, treated water is pumped through a UV disinfection bank providing 17 watts of emitted UVC radiation at 200 to 280 nm, to maintain maximum possible disinfection during storage.

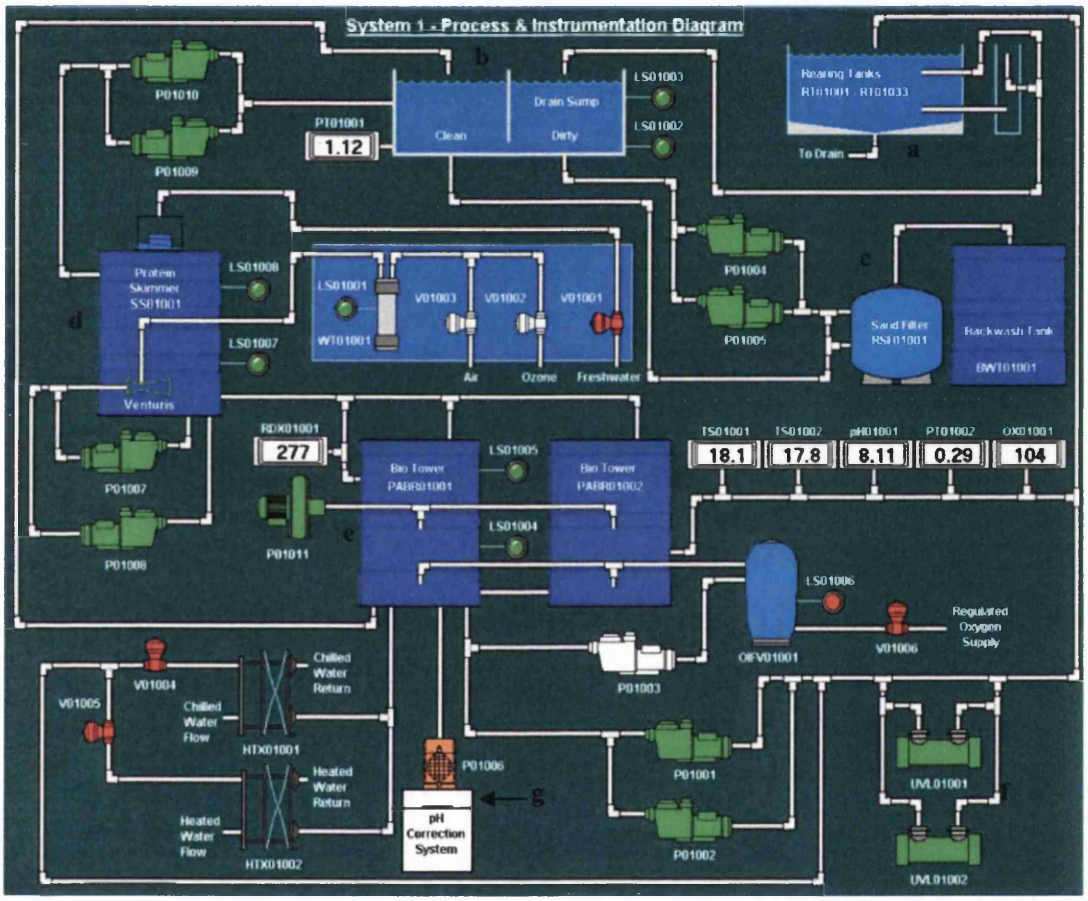

Figure 4: Screen shot of recirculating aquaculture system treatment cycle, showing livestock rearing tanks (a), drain sump (b), sand filter (c), protein skimmer (d), bio reactor (e), UV sterilisers (f) and pH correction/dosing system (g).

The configuration of the treatment system for the experimental RAS used for all of the fish feeding experiments is shown in Figure 4. Within the facility, water flows out of the livestock tanks (a) into a sump tank (b), from where it is pumped to a sand filter (c) providing a physical filtration stage. It is then passed into a protein skimmer

(d) to remove fine solids and organic detritus by foam fractionation, which also uses low level ozone dosing to help oxidise nitrites and organic material. Next the water is passed into a bioreactor (e) filled with biomedia colonised by nitrifying bacteria. Finally, from the biofilter the water is pumped through UV sterilisers (f) and back to the livestock tanks. The pH is controlled using an automated dosing system (g) which feeds measured doses of NaOH solution into the biofilter to maintain a constant predetermined pH. Of 60,000 l total system volume, approximately 3,000 to 6,000 (5 to 10%) is exchanged per week through filter backwashing. This waste water and the foam emanating from the protein skimmer is pumped to a series of effluent settlement tanks (c), from where the waste water supernatant is discharged to drain.

The RAS employed in this research has a total operating volume of 60 m^3, and consists of 30 blue circular polypropylene tanks (Fig. 5), each 1.4 m in diameter and having a 1.5 m^3 operating volume, in three rows of ten (row A, B and C). Each was equipped with a top and bottom inlet mounted on the wall of the tank, and a central top and bottom drain (Fig. 6). The waste water and solids, prior to returning to the treatment system, pass through a swirl separator allowing coarse solids to settle, from where they can be removed by the operator on a daily basis. Tanks were also brushed internally when required, and regularly washed down externally to maintain levels of cleanliness and hygiene.

Figure 5: Photograph showing the 1.4 m diameter livestock tanks in the experimental recirculating aquaculture system.

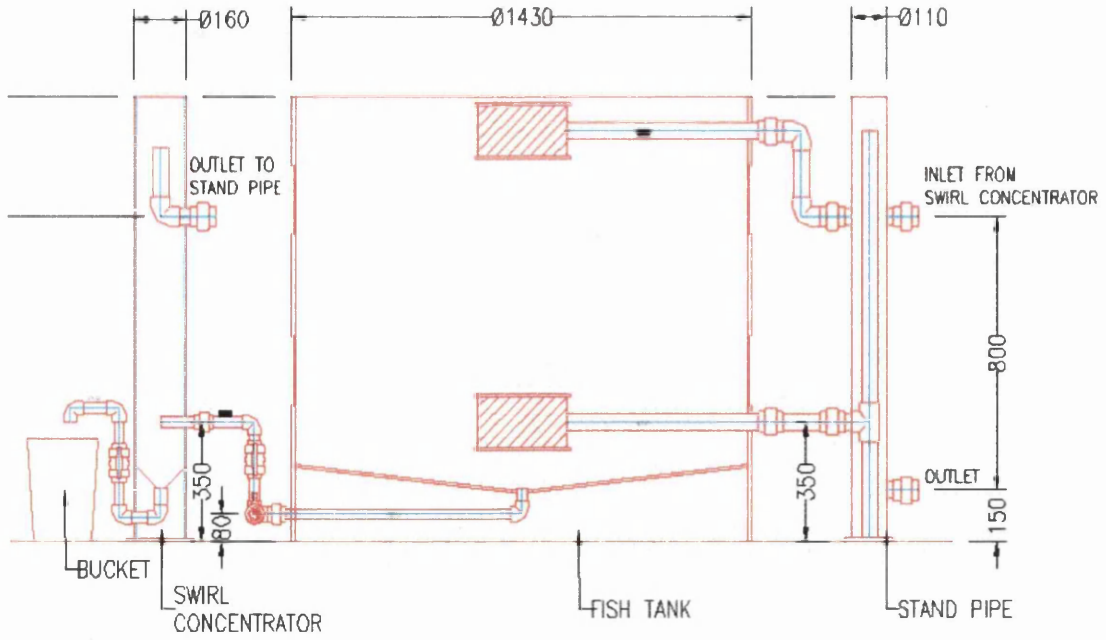

Figure 6: Schematic of the 1.4 m diameter livestock rearing tanks in the experimental recirculating aquaculture system.

2.2 System monitoring and record keeping

The system redox potential, pH and temperature were monitored in real time within the water treatment circuit, this data being logged centrally. Deviation of any water parameter outside of a set range prompted the system to alert members of staff to the issue for immediate attention. A twice daily manual check was also carried out and recorded of redox potential, pH, temperature, and the operation of the life support system.

Bi weekly water chemistry was also carried out on the system water and recorded by CSAR staff. This determined that the levels of ammonia, nitrate and nitrite were at safe levels. All water chemistry was carried using a Hach DR/2500 Colorimeter (Hach Company, USA) using the following protocols. Methods that were used are included in Appendix 1.

2.3 Experimental design for fish feeding experiments

The fish feeding experiments were designed with the help of a statistician to satisfy Home Office regulations in relation to numbers of animals used in experimentation, i.e. to minimize the numbers of fish used while retaining statistical power. The experiments were of a nested design, whereby tanks (replicates) were nested within

feeds. This allowed for the potential analysis of the significance of differences between tanks within feeds, and between the feeds themselves, making it possible to determine if effects were uniform across the replicated of an experimental feed. The experiments were carried out in rows of equal numbers of tanks where possible, with one replicate of each feed in each row, assigned randomly (see below).

To avoid bias in any study, each was carried out "blind" so that the experimental operator was unaware of which feeds were being administered to which tanks, by colour coding the feeds, and assigning these colour coded feeds randomly to tanks. This was achieved by assigning one replicate of each feed to a number between 1 and the total number of feeds (6, 7, 9 or 10 depending on the trial in question). An online random number generator (random.org) was then used to assign these numbers to the tanks in one of rows A, B or C sequentially. The experimental operator was made aware of which colour represented which feed only after the trial had ended and initial data analysis was complete.

2.4 Experimental feed production

All experimental feeds were produced at Skretting Feed Technology Plant (Stavanger, Norway) by way of extrusion (Wenger TX57, double screw) and drying (Wenger, 3 zones). An Yttrium oxide (Y_2O_3) premix (10% concentration) was added to the dry mix at 0.1% dietary inclusion as a marker to measure the apparent digestibility of nutrients in the feed, as validated in previous experiments (Austreng *et al.*, 2000, Carter *et al.*, 2003, Ward *et al.*, 2005). All feeds were produced as 3 mm and 4 mm pellets, to allow for the requirement of the fish for different pellet sizes as they grow. Specific feed formulae will be provided in detail for each stage of the experimental process.

2.5 Quarantine procedure and stock husbandry

To maintain biosecurity in the experimental RAS at all times, incoming livestock were stocked into a separate, biosecure 10 m^3 quarantine system and observed for clinical signs of infection for approximately 8, 13 and 13 weeks with 97.7, 98.4 and 88.4% survival respectively for the different batches of fish used in this research.

All stock animals were fed Skretting Europa© feeds of between 1.6 and 3.4 mm pellet diameter (55% protein, 15% oil, 12.5% ash, 7% moisture, 18.27 MJ/kg digestible energy) to excess on 12 hr timed belt feeders in the case of the first stock of cod and the sea bass, and by hand in two meals per day (am and pm) in the case of the second stock of cod. Uneaten food and faeces were removed by siphoning and emptying the waste collectors once in the morning every day.

2.6 Anaesthesia and euthanasia

The anaesthetic agent used in all trials was ethyl-3-aminobenzoate methanesulfonate salt solution ($C_9H_{11}NO_2 \cdot CH_4SO_3$, synonyms MS222 and Tricaine), supplied by Sigma Aldrich in powder form. A 10 ppt stock solution was made of this using 1 g the salt in 100 ml of water. This was stored at approximately 5°C and was diluted as required for the species of fish and for the procedure to be carried out.

In the case of the anaesthesia of European sea bass for short term procedures that required the animal to recover, a solution of 150 ppm (150 ml of stock solution or 1.5 g of the salt in 10 litres of system water) was prepared. The animal was immersed in the anaesthetic solution for approximately 3 min until it had lost its balance, at which time it was removed in order to carry out the required procedure. Following this, the fish was placed in clean system water. In a further 3 to 5 min the fish had recovered and resumed normal behaviour. In the case of the fish not being required to recover, i.e. in euthanising the animal, the fish was placed in a slightly stronger anaesthetic solution (250 ppm) and was not removed until cessation of ventilation was observed, upon which time the fish was dispatched with a sharp blow to the back of the head followed by physical destruction of the brain (pithing) and immediately placed in a freezer.

In the case of the anaesthesia of Atlantic cod for short term procedures that required the animal to recover, a solution of 50 ppm (50 ml of stock solution in 10 litres of system water) was prepared. The animal was immersed in the anaesthetic solution for approximately 3 min until it had lost its balance, at which time it was removed in order to carry out the required procedure. Following this, the fish was placed in clean system water. In a further 3 to 5 min the fish had recovered and resumed normal behaviour. In the case of recovery not being desirable, i.e. in euthanising the animal,

the fish was placed in a slightly stronger anaesthetic solution (150 ppm) and was not removed until cessation of ventilation was observed, upon which time the fish was dispatched with a sharp blow to the back of the head followed by pithing and immediately placed in a freezer.

2.7 Calculation of growth and performance parameters

The main growth parameters and performance indicators were calculated according the equations listed below.

Body weight increase g day^{-1}:

$$= \frac{\text{(final body weight - initial body weight)}}{\text{days}}$$

Specific growth rate (SGR) % b.wt. day^{-1}:

$$= \frac{\text{(ln (final body weight) - ln (initial weight) * 100}}{\text{days}}$$

2.8 Calculation of feed intake and feed conversion ratio

Different feed intake measurement methods were used for the different studies, and these are therefore described in chapter specific methodologies. The calculations used for the parameters were however the same, as shown below.

Feed intake % b.wt. day^{-1}:

$$= \left[\frac{\left[\frac{\text{intake per fish}}{\text{days}} \right]}{\text{(final body weight x initial body weight)}} \right]^{0.05} * 100$$

Feed conversion ratio (FCR):

$$= \frac{\text{final body weight - initial weight}}{\text{total individual feed intake}}$$

2.9 Analysis of gastrointestinal morphometrics and blood chemistry

Gastrointestinal morphometric analysis was carried out by two MSc students at Swansea University in the latter parts of 2006 and 2007. The methods (shown in Appendix 3) and subsequent results reported herein are adapted from their dissertations (Buckley, 2006, Castleman, 2007).

2.10 Feed and ingredient digestibility

Digestibility of each nutrient or feed fraction i.e. protein, dry matter and organic matter for each feed was determined at the Skretting Aquaculture Research Centre (Stavanger, Norway) and was calculated according the following formula (Lupatsch *et al.*, 1997):

$$\text{Digestibility (\%)} = 100 - \left\{ 100 * \left(\frac{Y_2O_{3Feed}}{Y_2O_{3Faeces}} \right) * \left(\frac{Nutrient_{Faeces}}{Nutrient_{Feed}} \right) \right\}$$

2.11 Proximate analysis of feeds and fish carcasses

Proximate compositions of feeds and fish whole bodies were carried out in the laboratory at Skretting Aquaculture Research Centre, Norway, according to the protocols described in Appendix 2. Whole fish proximate samples were prepared in Swansea prior to shipping by grinding the frozen carcasses of six fish per tank using a proprietary meat grinder twice until a paste like consistency was achieved. A sample of this paste was then dried to constant weight in pre dried and weighed crucibles at 105°C to determine the moisture and dry matter content. This sample was then placed in a muffle furnace at 550°C for 12 hr to determine the ash and volatile compound content. The remainder of the paste was spread evenly in large foil oven trays and dried to constant weight at 105°C, after which it was ground to a powder using a proprietary kitchen blender. It was this ground and dried sample that was dispatched to the Skretting Aquaculture Research Centre, Norway, for analysis. To maintain accuracy of the results, a sub sample of this material was also dried as above to ensure the absolute moisture content was known for the ensuing calculations.

2.12 Faecal particle size distribution

2.12.1 ***Method development.*** Non-trial Atlantic cod (*Gadus morhua*) were fed on a commercially available extruded pellet, Skretting Europa©. On the day of stripping, a number of fish of approximately 150 g were netted from their holding tanks and anaesthetised to the point of loss of balance (see Chapter 2, Section 2.6 for anaesthesia procedure). They were then removed from the anaesthetic and were stripped of the contents of the posterior portion of the gastrointestinal tract (GI tract) – henceforth referred to as faeces – by applying a gentle pressure slightly anterior to the vent using forefinger and thumb, working towards the posterior of the fish. The faeces collected from each fish were pooled into a 30 ml Sterilin tube, and fish were repeatedly stripped over several weeks. The fish were then returned to their holding tank and observed for safe recovery.

Stripping was used as a method for faeces collection due to the requirement for whole, undiluted faecal waste for certain analyses, for example rheological analysis. Analysis of outlet water would have provided information on the actual particle size distributions of faecal material after being voided and exposed to system water for some time. However, in this case, the interest was in the faecal material itself and effects of experimental diets on that material, with later reference to its behaviour in the system water. Rheological analysis would have been impossible on faeces so diluted by water so as to be individual suspended particles in water, as the material would have shown behaviour and properties no different to water itself. The reasoning for the selection of stripping as a method for faecal collection is further discussed in Chapter 3, Section 3.1.

In order to determine whether freezing had any measurable differences on particle size distribution and therefore whether faeces could be frozen between samplings prior to analysis, some of the fresh raw faecal samples were immediately frozen at -20°C. The fresh material was analysed for particle size distribution, and further samples were placed in crucibles and dried to a constant weight at 105°C. A known volume of fresh faeces was also mixed with a known volume of artificial seawater (prepared using distilled water and Tropic Marin® artificial sea salt, Tropic Marin GmbH, Germany) with salinity 32 ppt, to create an aqueous mix of suspended faeces particles. This was done to enable the filtration of the faeces and to determine what

effect this had upon faecal recovery. The mixture was analysed for particle size distribution, and volumetric samples were placed in crucibles and dried to a constant weight at 105°C to determine the mass of faeces recovered at each stage as a percentage of the original sample. The remaining mixture was then filtered through a 500 μm nylon mesh filter and then through a 250 μm nylon mesh filter, with the particle size distribution and dry weight sampling process repeated at both stages. This process was repeated with the faeces that had been frozen for 24 hr to determine the effect of freezing.

This process consisted in essence of a serial or sequential filtration system. It is appreciated that this process can have an effect on filtrate particle size distribution, as demonstrated by Langer *et al.* (1996) who showed that drum filtration changed the particle size distribution of aquaculture wastewater, resulting in increasing amounts of the smaller particle fractions, and thereby indicating a partial breakdown of larger particles during the filtration process. Droppo *et al.* (1995) have also discussed the fact that serial filtration is not an accurate way of fractionating sediments. However, as fractionation was not used to determine experimental particle size distributions but instead used in developing methods, the effect is relatively inconsequential. It is also generally agreed that a pressure of 10 cm (water column) should not be exceeded in order to avoid excessive particle extrusion through the filter mesh, and this condition was respected for all filtration processes.

Particle size distribution analysis was carried out using a Malvern Mastersizer© (Malvern Instruments Ltd, UK) located in the Multidisciplinary Nanotechnology Centre in the School of Engineering, Swansea University. In order to determine the accuracy of the particle size distributions obtained, silica control particles of known size were run though the machine during the same session.

Dry weight. This simple method was used to determine the amount of faeces that could be recovered following filtration to remove coarse particles from the faeces as required for subsequent analysis. Differences in particle size between fresh and frozen faeces as determined by dry weight of fractions recovered following filtration were minimal (Fig. 7), enabling the data from fresh and frozen material to be combined. After mixing the faeces with artificial seawater, 90.1 ± 11.6% (n = 8) of

the original mass of faeces was recovered. Following filtration through a 500 µm mesh, 56.2 ± 4.2% of the original sample remained in the mixture. Following filtration of this mixture through a 250 µm mesh, 37.7 ± 9.9% of the original sample remained. Moisture content of the unfiltered faeces again showed minimal difference between fresh and frozen samples, the combined value being 83.9 ± 0.3%, n = 8.

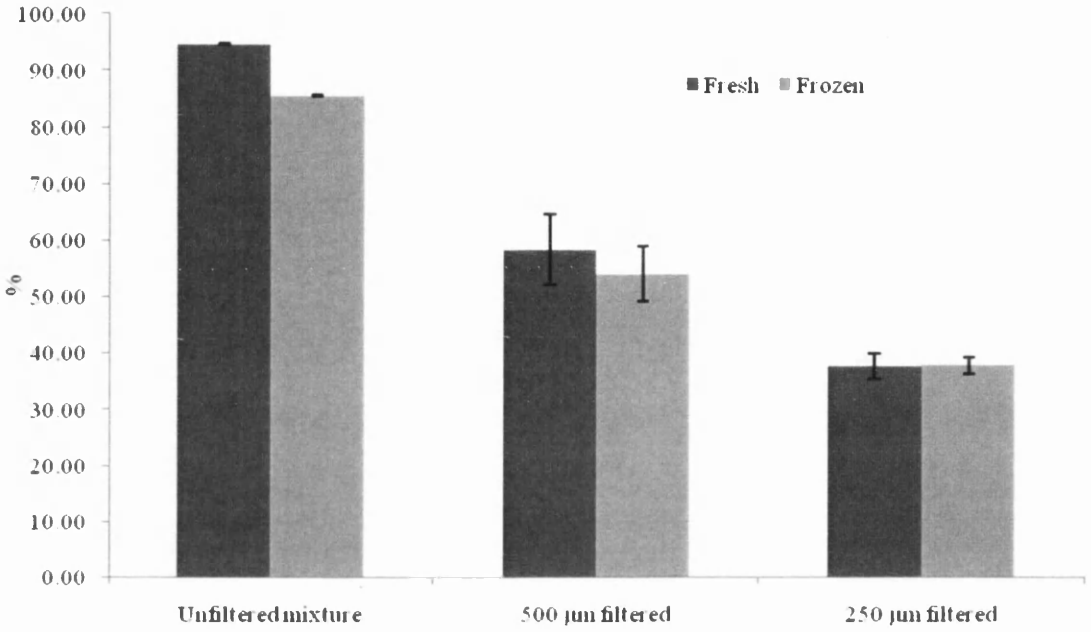

Figure 7: Bar chart showing dry weight of the unfiltered water/faeces mixture, and of the mixture following 500 µm and 250 µm filtration, all as a % of the original dry faecal weight (mean ± standard deviation); n = 4.

These results show that using a 250 µm mesh to remove coarse particles would necessitate the collection of approximately 2.65 g of untreated faeces to yield 1.00 g of sample for analysis (i.e. 62% of the faeces is lost during filtering).

Particle size. The particle size distributions taken from the raw faeces and the faeces/water mix (Fig. 8) show that the mixture was representative of the raw faeces. Furthermore there were no major differences between the particle sizes from fresh and frozen faeces samples at any stage in the filtering process (Figs. 8, 9 & 10) enabling faeces to be frozen following stripping for subsequent analysis, without altering the particle size distribution characteristics.

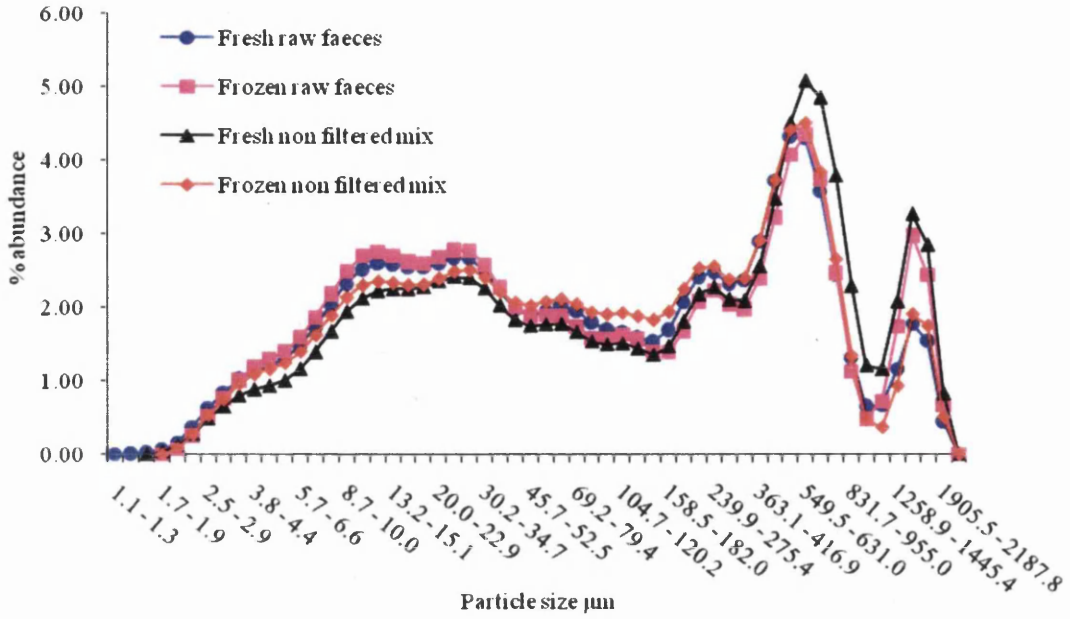

Figure 8: Particle size distribution curves for fresh and frozen faeces and faeces/water mixture.

It was observed that particles larger than the stated mesh aperture were detected in filtrate from both the 500 and the 250 µm filters (Fig. 9 & 10). Approximately 2.37% of the particles observed following 500 µm filtration fell in the ranges > 500 µm (maximum range into which some particles fell = 724.4 - 831.7 µm) and approximately 2.79% following 250 µm filtration were > 250 µm (maximum range into which some particles fell = 631.0 - 724.4 µm).

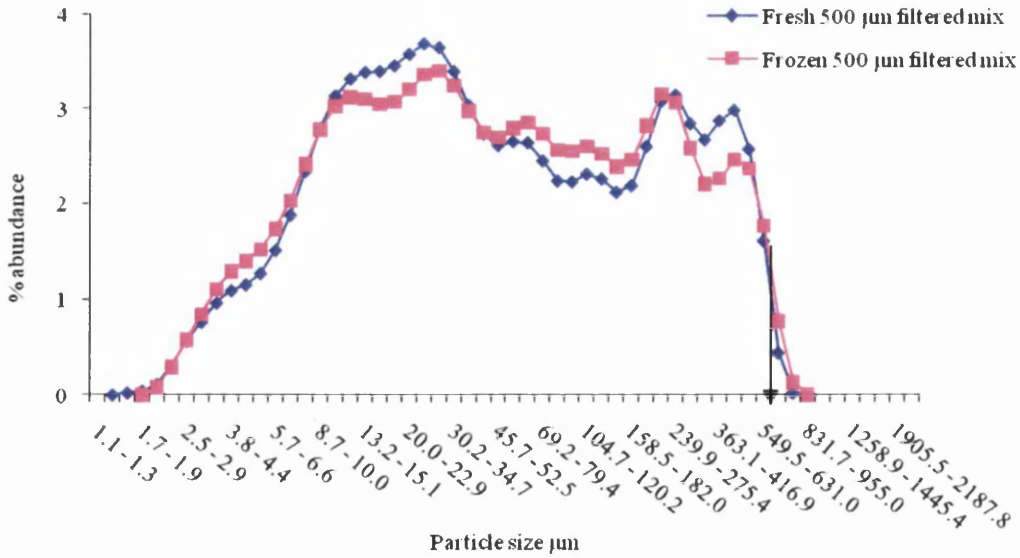

Figure 9: Particle size distribution curves for fresh and frozen filtrate following filtering of the faeces/water mixture through a 500 µm nylon mesh. Vertical arrow shows approximate position of the 500 µm size class.

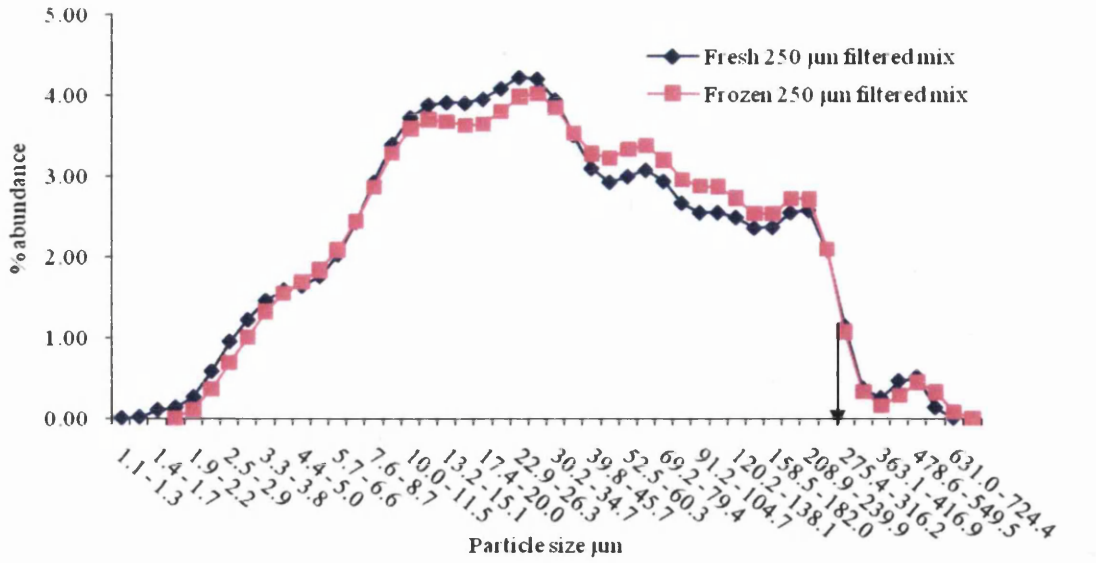

Figure 10: Particle size distribution curves for fresh and frozen filtrate following filtering of the faeces/water mixture through a 250 µm nylon mesh. Vertical arrow shows approximate position of the 250 µm size class.

In order to determine whether these larger-than-expected particle sizes following filtration were due to imperfections in the mesh apertures, rather than error borne from improper cleaning of the equipment (i.e. coarse particles remaining following a previous run) or post filtration coagulation, a further analysis was carried out using particles of known size. If the seemingly large particles were due to improper cleaning one would expect to see similar-sized particles in all analyses; the remaining particles for the previous run. If they were due to larger-than-specified mesh apertures one would expect the mesh to let through a significant amount of particles with dimensions greater than the specified aperture size in filtered mixes, e.g. > 250 µm when filtered through imperfect 250 µm mesh.

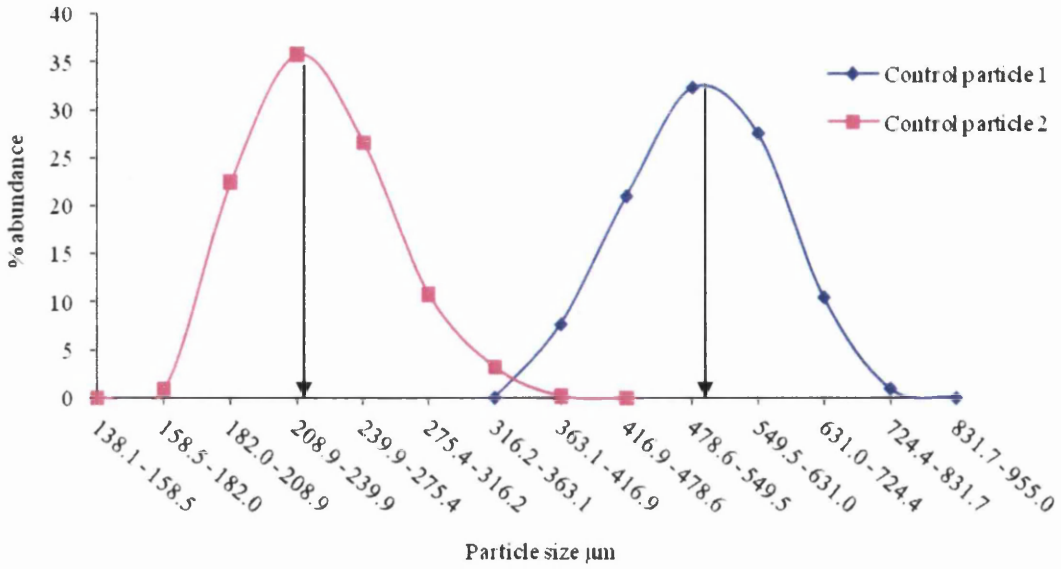

Figure 11: Particle size distribution curves for two control particles of known size (1 = 500 µm, 2 = 250 µm). Vertical arrows show approximate positions of respective size classes.

Particle size distributions for 2 types of particle with known dimensions showed normal distribution curves around expected particle size (Fig. 11) demonstrating that there were no carry-over of coarse particles in the Malvern Mastersizer© between runs. When the standardized 250 µm and 500 µm particles were mixed and filtered through a 250 µm mesh, just 3.9% of particles in the filtrate were > 250 µm (with a size range of 363.1 - 416.9 µm), demonstrating that none of the 500 µm particles had made their way into the filtrate, and that the mesh was therefore accurate in its specification.

Having ruled out carry-over of particles between runs and larger-than-specified apertures in the 250 µm mesh, it is concluded that particle interactions occurred in the faeces/water mixture following filtration, leading to combined particles that were significantly larger than the filter aperture size. It was notable that the maximum particle size was similar in both the 500 and 250 µm filtrates. This suggests that the same attractive forces between similar-sized particles were affecting both filtrates, i.e. finer particles that would possess the greatest attractive forces (high surface area to volume ratio) and form oversized flocs. A similar phenomenon was reported by Brinker *et al.* (2005c) in aquaculture waste water after being filtered through an 80 µm drum filter, and was a significant property that could be exploited to increase the filtration efficiency of the physical filters.

In summary, the method development for faecal particle size distribution confirmed that it is possible to store faeces in frozen form until measurement, with negligible change in their particle size properties, and also that faecal particle size distribution can be determined using the methods shown.

2.12.2 ***Final method.*** Fish faeces were analysed for particle size distribution in the Multidisciplinary Nanotechnology Centre, School of Engineering, Swansea University, using a Malvern Mastersizer©. In order to ensure that the suspension of particles was well mixed, an electric mixing vessel was employed, which also introduced the sample to the instrument. A small volume of sample, approximately 0.25 g, was injected into the mixing vessel, along with enough distilled water to ensure the laser obscuration as a result of the suspension was within the required range for the instrument to operate.

The measurement cycle automatically took two measurements of particle size distribution for each sample, and supplied the data as a mean of these two values. This was carried out for each sample (i.e. each tank). The data were then expressed as a plot of the mean of the three replicates for each feed at each range. In the case of the Phase 2 experiments, particle size was analysed by Wallace (2008), as part of an MSc. dissertation.

pH was not tested for in the suspensions used for particle size distribution analysis. There is a potential therefore that particle size distribution could have been influenced by pH differences in the suspensions, through the mechanisms described in Chapter 2, Section 2.13, below. It is also possible that the mixing vessel itself could have had an effect on the particle size distribution. However, as all samples were treated in the same way, the effect of these on potential similarities or differences between samples is considered to be minimal. However, in future research, it would be desirable to ensure that pH was recorded at every stage, in order to fully account for this potential source of error. Also it may be desirable to ensure that particle sizing methods are further validated during the process to ensure repeatability and comparability between studies.

2.13 Faecal zeta potential

2.13.1 ***Method development.*** Analysis of Uε, the value that used to calculate ζ, was carried out using a Malvern Zetasizer© (Malvern Instruments Ltd, UK). Samples of the frozen cod faeces collected previously were used for this experiment (see Chapter 2, Section 2.12.1). In order for accurate measurements of mobility to be made, samples of suspended particles must be at a final concentration of 0.4 g l^{-1}, so as not to hinder the mobility of particles through the aqueous medium and not to clog the fine vessels within the instrument. As the particles must also be suspended for this analysis, there was no requirement for those particles that might ordinarily rapidly settle out of suspension to be present, therefore a 4 g l^{-1} suspension of fine faecal particles in water was prepared for analysis.

For analysis of mobility versus pH, a 2 g sample of liquid faeces was filtered through a 250 μm nylon mesh filter, and washed through with 500 ml of 32 ppt artificial seawater prepared as outlined previously. In the case of mobility versus salinity analysis, a 2 g sample of liquid faeces was filtered through a 250 μm nylon mesh filter, and washed through with 500 ml of artificial seawater made up to 10, 15, 20, 25, 30, 35 and 40 ppt. The resulting fine faecal particle suspensions were then ready to be analysed for Uε.

Conical flasks were prepared with 135 ml of the same 32 ppt artificial seawater, and adjusted to one of seven pH values (3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0) using HCl and Na_2OH. The ability of the solutions to buffer the pH was not deemed necessary in this instance as it was a method development exercise and was not to produce data to be used for analysis. A further set of flasks was prepared with 135 ml of artificial seawater made up to 10, 15, 20, 25, 30, 35 and 40 ppt. To create the final faeces in water suspensions at the required concentration, 15 ml of the 4 g l-1 mixtures was dispensed into each of the relevant flasks and stirred. Each suspension was then injected into the instrument in order.

Determination of Uε was performed at 23IC (water Er = 79.2690, υ = 0.9327), and ζ was calculated as follows:

$$U\varepsilon = \frac{Eo\ Er\ \zeta}{\upsilon}$$

Where Uε is the Uε of the particle, Eo is the permittivity of free space (8.854187818 x 10-12 Fm^{-1}), Er is the dielectric constant and υ the viscosity of the continuous phase (known at the operating temperature), and ζ is the zeta potential.

This equation is rearranged in order to calculate ζ from Uε:

$$\zeta = \frac{\upsilon\ U\varepsilon}{Eo\ Er}$$

Twenty five measurements were taken for the mobility of fine faecal particles at each pH and salinity value. The first 15 of these measurements were taken for instrument stabilisation purposes and discarded, and the mean of the final 10 measurements was taken as the value of Uε. This value was then used to determine ζ according to the above equation.

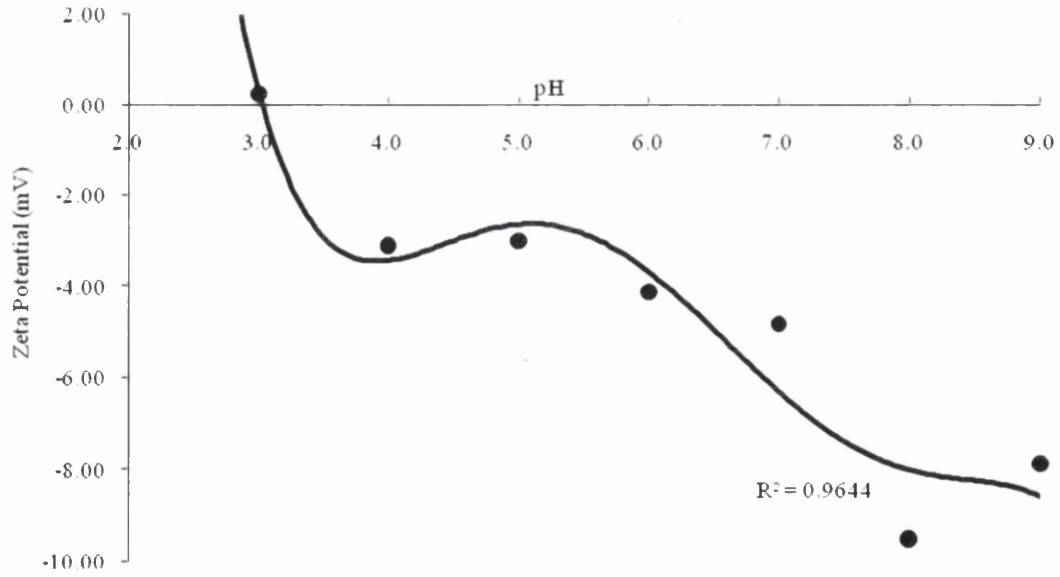

Figure 12: Effect of pH on the zeta potential of a suspension of fine (< 240 μm) Atlantic cod faecal particles. Plot fitted with 5 order polynomial trend line.

The surface charge of the fine faecal particles was negative for the majority of the pH range employed in this study (Fig. 12). The IEP was achieved at pH 3.1. There was a sharp drop in zeta potential between pH 3.1 and pH 4.0, from a charge of 0.0 to -3.0

mV. Between pH 4.0 and pH 5.5 the zeta potential reached a plateau at between -3.0 and -3.5 mV, followed by a rapid decrease to a low of -11.0 mV at pH 8.0, after which point it appeared to plateau again.

There was also a slight change in ζ of around 6 mV with a change in electrolyte concentration at pH 8.3 (Fig. 13). The ions in the electrolyte are known as the indifferent ions as these do play a direct part in determining the charge on the surface of the particle. In reality, the indifferent ions act to compress the diffuse double layer, leading to a smaller repulsion force between the particles (Hunter, 1993) and the absolute value of the Uε decreases due to the screening effect of electrolyte ions on the electrical potential at the surface of shear (Valle-Delgado *et al.*, 2003).

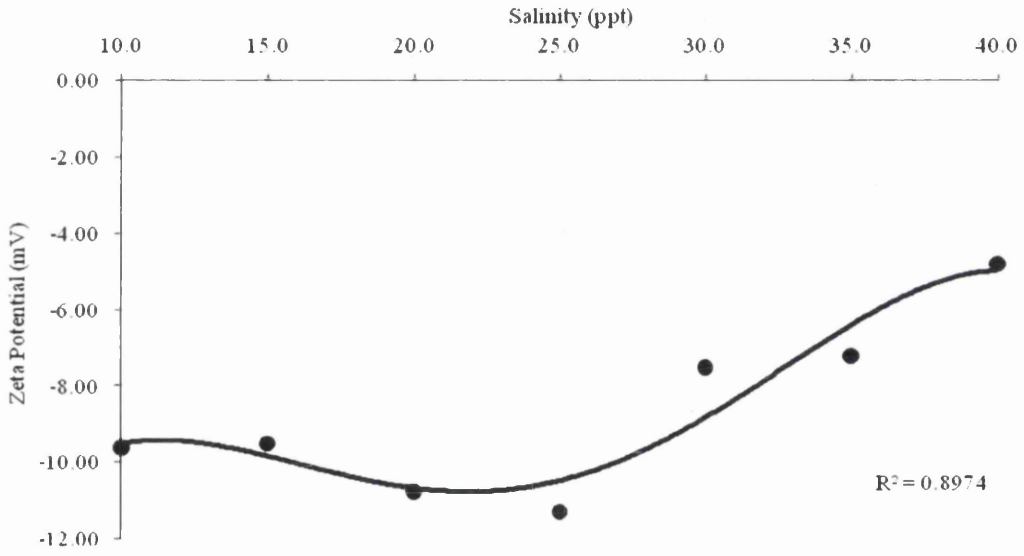

Figure 13: Effect of salinity on the zeta potential of a suspension of fine (< 240 μm) Atlantic cod faecal particles. Plot fitted with 4 order polynomial trend line.

These tests confirm that the methods described are appropriate for zeta potential measurement of fish faeces and that, with a great enough range of conditions, an IEP can also be determined.

2.13.2 ***Final method.*** A series of solutions of known properties were prepared to analyse the effect of pH on the Uε of fine faeces particles in an aqueous medium. The pH values to be tested were 3, 4, 5, 6, 7, 8, 9, 10, and 11, made as 32 ppt seawater buffers. It is clear from the initial findings that pH plays an important role

in determining the ζ, and it for this reason that it was ensured that the pH solutions were buffered.

Buffers were prepared from methods adapted from Robinson (1955) and as shown in Table 2. Adjustment was made using the relevant compound for the addition of NaCl, with pH and salinity being measured and monitored using a WTW 330i handheld meter (WTW GmbH, Germany). Any sediment was removed by vacuum filtering through glass fibre filters.

Table 2: Solutions used to prepare a series of buffers for zeta potential analysis. Buffers were made as seawater buffers, and were adjusted using the relevant solution to the required pH and salinity (32 ppt).

	Solution 1			**Solution 2**		
pH	**Common name**	**Formula**	**Volume ml**	**Common name**	**Formula**	**Volume ml**
2	Potassium chloride	KCl (0.2M)	50	Hydrochloric acid	HCl (0.2M)	13.0
3	Potassium hydrogen phthalate	$KHC_8H_4O_4$ (0.1M)	100	Hydrochloric acid	HCl (0.1M)	44.6
4	Potassium hydrogen phthalate	$KHC_8H_4O_4$ (0.1M)	100	Hydrochloric acid	HCl (0.1M)	0.2
5	Potassium hydrogen phthalate	$KHC_8H_4O_4$ (0.1M)	100	Sodium hydroxide	NaOH (0.1M)	45.2
6	Monopotassium phosphate	KH_2PO_4 (0.1M)	100	Sodium hydroxide	NaOH (0.1M)	11.2
7	Monopotassium phosphate	KH_2PO_4 (0.1M)	100	Sodium hydroxide	NaOH (0.1M)	58.2
8	Monopotassium phosphate	KH_2PO_4 (0.1M)	100	Sodium hydroxide	NaOH (0.1M)	93.4
9	Borax	$Na_2B_4O_7.10H_2O$ (0.025M)	100	Hydrochloric acid	HCl (0.1M)	9.2
10	Borax	$Na_2B_4O_7.10H_2O$ (0.025M)	100	Sodium hydroxide	NaOH (0.1M)	36.6

The dilute faeces samples were prepared using distilled water to prevent untoward effects of the aqueous medium prior to adding the dilute faeces sample to the solution with the desired salinity or pH value. This also enabled the best use to be made of the very small volumes of liquid faeces available. The liquid faeces were filtered through 250 µm nylon mesh filters, and rinsed through with a volume of distilled water to create dilute mixtures of approximately 4 g l^{-1}, i.e. 0.25 g of faeces rinsed through with 62.5 ml of distilled water (exact concentration was not critical as long as the mixture has enough particles to measure, but not too many to cause

significant settlement problems). These suspensions were then added to a flask of the prepared pH solution, to create a suspended mixture of 0.4 g l^{-1} of faeces in water (i.e. 3.0 ml of faeces/water mixture in 27.0 ml of the required solution). This final mixture was stirred for 5 min to allow the particles to be suitably affected by their new chemical environment, and was then injected in to the Malvern Zetasizer©. As in the method development stage, determination of Uε was performed at 23°C (water Er = 79.2690, υ = 0.9327), and 25 measurements were taken for the mobility of fine faecal particles at each pH and salinity value. The first 15 of these measurements were taken for instrument stabilisation purposes and discarded, and the mean of the final 10 measurements was taken as the value of Uε. This value was then used to determine ζ according to the above equation. The result was a series of values for the faeces resulting from each feed for pH, from which means can be taken of the replicates and curves drawn as in the method development stage. In the case of the Phase 2 experiments, ζ was again analysed by Wallace (2008).

To these plots polynomial trend lines were fitted that best described the data (ranging from 3 Order to 5 Order). This resulted in a polynomial equation of the form:

$y = a(x^5) + b(x^4) + c(x^3) + d(x^2) + e(x^1) + f$ (5 Order) OR

$y = a(x^4) + b(x^3) + c(x^2) + d(x^1) + e$ (4 Order) OR

$y = a(x^3) + b(x^2) + c(x^1) + d$ (3 Order)

Into these formulae were entered the average value for the pH of the experimental rearing system (x = 8.05). This resulted in a value for the ζ of the faecal sample at the system operating pH for each replicate, which could then be compared between feeds. It is noted that in this methodology, ζ was measured after the faeces samples were suspended in the buffer for approximately an hour, whereas particle size distribution was measured after a much shorter time in suspension. The effect of suspension in the case particle size distribution would probably have altered the particle size distribution to some extent, but this was not tested for. However, all experimental protocols remained identical between samples, and so differences due to this phenomenon would have been negligible.

2.14 Faecal dry matter

The measurement of the dry matter of faeces was simply achieved by placing a known volume of wet faeces (volume depended on volume available for all analyses) on a pre-dried and pre-weighed piece of tinfoil. This was then placed in a drying oven at 105°C until constant weight was achieved. The foil were then removed from the oven and cooled to room temperature in a desiccator, at which point they were weighed and the loss in weight recorded as moisture content in %, which was simply converted to dry matter by subtracting from 100. This data are used in order to calculate other parameters and is not reported directly.

2.15 Faecal production

The total mass of faeces produced relative to the mass increase of the fish (faecal production, g kg^{-1} growth) by the experimental fish on different feeds was calculated using the digestibility data determined using the formula and methods in Chapter 2, Section 2.10 (DM = dry matter).

$$\text{Faecal production g kg}^{-1}\text{ growth} = \left\{ \text{DM FCR} * \left[\frac{100 - \text{DM digestibility}}{100} \right] \right\} * 1000$$

Having determined this value per feed it is possible to incorporate the particle size distribution data to calculate the production of faeces in any given size range. This is achieved by determining for the distribution in question what proportion of the distribution lies in any particular range. In this case, the proportion of the distribution up to approximately 100 μm and 250 μm has been determined and the production of faeces in these ranges has been calculated. These values were arrived at due to it being accepted that particles of up to 100 μm are those that dominate the water in a RAS (Chen *et al.*, 1994), and due to 250 μm being the mesh size which was used to remove 'coarse particles' for ζ and rheological analysis.

2.16 Faecal density and settling

In order to determine the settling velocity of the faecal particles, a value for the density of faecal particles was required. Determination of this was attempted in the laboratory, but the results were not reproducible and so a value was taken from the literature. This value was determined as the mean of those derived from a range of

published and cited sources, and was calculated to be 1102 kg m^3 (Chen *et al.*, 1993, Patterson *et al.*, 2003) which is also the value determined by Patterson *et al.* (2003) from their own research.

Using Stokes' Law for the frictional force exerted on very small spherical objects in a continuous viscous fluid (equation 1), the terminal velocity and thus the settling rate can be calculated (equation 2).

1. $$F_d = 6\pi \mu R V$$

Where F_d is the frictional force (N), μ is the fluid's dynamic viscosity (Pa s), R is the radius of the spherical object (m), and V is the particle's velocity (m/s).

2. $$V_s = \frac{2}{9}\frac{(\rho_p - \rho_f)}{\mu} g R^2$$

Where V_s is the particles' settling velocity (m/s), g is the gravitational acceleration (m/s^2), ρ_p is the mass density of the particles (kg/m^3), and ρ_f is the mass density of the fluid (kg/m^3).

This final equation was used to calculate the time taken for particles to settle in 10 cm of water, to allow standardisation of data across studies. In order for this data to be calculated, the particle size distribution of each replicate of the experimental faeces from any particular feed was expressed as a cumulative distribution starting from the coarsest particles at 1% to the finest particles at 100%. From this, the minimum particle size was taken at set fractions (i.e. 10, 50, 60 70 80 90 and 95%) of the distribution. These particle sizes were then fed into the equation 2 as the value of R and a settling velocity was determined at each fraction.

3. $$\text{Time taken to settle in 10 cm of water} = \frac{10}{V_s}$$

This settling velocity was used in equation 3 to determine the time taken in s to settle in 10 cm of water at each fraction, from which a plot was drawn.

2.17 Rheology

2.17.1 ***Method development.*** Based on preliminary visual observation of the Atlantic cod faeces collected as described in Chapter 2, Section 2.12, the material appeared to be a suspended solid or slurry type material. A general difficulty in testing such fluids in a rotational rheometer related to coarse solid particles (> approximately 800 μm) directly blocking the gap between the stationary and rotational plates of the rheometer, or finer particles (> 250 μm) rolling over one another, 'piling up' and thereby also blocking the gap. Such events would be registered by the very sensitive instrument as sudden massive increases in viscosity, and as such would render the data meaningless. To circumvent these problems, the faeces were therefore filtered to remove coarse particles prior to analysis. This was achieved by passing the raw material through a 250 μm nylon mesh filter, and rinsing it through thoroughly with fresh distilled water. The resultant filtered mixture was then centrifuged at 2666 *g* for 15 min to ensure that all particles in the mixture were settled out of suspension. The supernatant was then decanted off and discarded. Some of this material was used to determine the moisture content of the filtered material, which was then compared to the moisture content of the raw faeces. If these values differed, then distilled water was added to the filtered and centrifuged particles to increase their water content to match that of the raw faeces. The reconstituted fine faecal sludge samples were then frozen at -30°C pending analysis.

In order for dynamic rheological testing to be carried out, the material must possess a linear range of stress, a frequency-independent range of stresses at which the material exhibits 'unchangeable' characteristics, i.e. no change in its viscoelastic properties. From this linear range, a strain can then be identified at which to analyse the material using a frequency sweep. To test whether this was feasible with the material in question, stock faeces collected from European sea bass was analysed using a Bohlin Gemini 200 Advanced Rheometer (Malvern Instruments Ltd, UK; serial number 04/200-050/CUR/C/101) controlled by the Bohlin R6.50.5.7 control program, located in the Institute of Life Sciences at Swansea University. Temperature was controlled by a Bohlin Peltier (Malvern Instruments Ltd, UK), and a 65 mm stainless steel vapour trap was employed to minimise evaporation. An amplitude sweep was carried out using a 40 mm short shaft stainless steel parallel plate (the rheometer cell

geometry), with a total test time of 651 s (31 samples), a steady shear rate of 0 1/s, a minimum strain of 0.0005, a maximum strain of 0.5, and an initial stress of 0.5 Pa.

These tests demonstrated that it was possible to find a limited linear range for reconstituted faecal sludge from European sea bass, enabling samples to be tested both dynamically and for simple viscosity (thereby ensuring that at least the minimum data would be available for every sample, should the dynamic testing fail for any reason).

2.17.2 ***Final method.*** Using the methods developed above, the raw faecal material was passed through a 250 μm nylon mesh filter, and rinsed through thoroughly with fresh distilled water. The resultant filtered mixture was then centrifuged at 4000 *g* for 15 min and the supernatant decanted off and discarded. Some of this material was used to determine the moisture content of the filtered material. The reconstituted fine faecal sludge samples were then frozen at -30°C pending analysis.

As in the method development stage, a Malvern Instruments Bohlin Gemini 200 Advanced Rheometer (serial number 04/200-050/CUR/C/101) controlled by the Bohlin R6.50.5.7 control program was used. Temperature was controlled by a Bohlin Peltier, and a 65 mm stainless steel vapour trap was employed to minimise evaporation. To ensure that a suitable strain was determined from within the linear viscoelastic range of the material, amplitude sweeps were carried out on the samples from within a trial with the largest volume of material available to test. An amplitude sweep was carried out using a 40 mm short shaft stainless steel parallel plate, with an initial resting phase of 60 s for thin samples and 300 s for thick samples was employed prior to the test to ensure the material was at a steady state. Following from this, the test was carried out with a total test time of 651 s (31 samples), a steady shear rate of 0 1/s, a minimum strain of 0.0005, a maximum strain of 0.5, and an initial stress of 0.5 Pa. Following this test, the sample was discarded.

Frequency sweeps were carried out at the predetermined strain on the same instrument. A resting phase of 60 s for thin samples and 300 s for thick samples was employed prior to the test to ensure the material was at a steady state. Following from this, the total test time was 1944 s (31 samples), with a minimum frequency of

0 .01 Hz, a maximum frequency of 10 Hz, and an initial stress of 0.1 Pa. Following this a simple viscometry test was also carried out on the same sample with a total test time of 310 s (31 samples), a minimum shear rate of 0.2 1/s and a maximum shear rate of 200 1/s.

2.18 Data handling and statistical data analysis

Statistical analyses were tailored to the properties of the dataset tested, and were carries out using SPSS v 16.0 (SPSS Inc, USA). All datasets were tested for normality using a Shapiro-Wilk test, agreed by most authors to be the most reliable test for non-normality for small to medium sized datasets of 3 to 50 data points (Royston, 1982, Royston, 1995). Normality was tested in larger datasets by the Kolmogorov-Smirnov test. In data sets containing more than 2 data points (replicates) in every group, Levene's Test of Equality of Error Variances was also used where possible. If data sets were found to be normal the parametric course of action outlined below for each parameter was followed. If the data were determined to be non-normal, firstly a transformation was attempted. If this was unsuccessful, a non-parametric Kruskal-Wallace test was used to determine whether statistically significant differences were present in the dataset, followed by pairwise Mann-Whitney U test to determine where those differences lay.

Where possible the nested design of the experiment was carried through to the analysis of the resulting data by utilising a 2 way nested ANOVA with a Duncan post-hoc test for statistically significant differences. In these cases an ANCOVA analysis was also used to verify the effect of the nested variable. Such datasets were those in which individual fish data were available, i.e. hepatosomatic index. Thanks to the larger size of these data sets, a GLM III model to test between subjects effects could also be used to ensure than the regression slopes were homogeneous, i.e. each group showed the same data trend. However, in the majority of cases this approach was not possible due to the final data resulting from start values which were not individual specific. For example, where bulk tank biomass was taken as a start value and individual weight as end values, the end values were averaged to produce one start and one end value per tank.

In the case of parameters in which one data point was produced per tank (3 per group), a 2 way ANOVA was carried out with a Duncan post-hoc test if statistical significance between feeds was found. In the case of datasets with limited data points (less than 3 for all or most groups) the upper and lower confidence limits of the 99% confidence interval was determined, and depending on the size of the interval, the data were tested further as above or simply described.

Intestinal morphometrics and blood chemistry data were analysed by the authors of the respective dissertations. Buckley (2006) initially tested each intestinal parameter within a fish pairwise using a paired t test where all assumptions of the test were met; data were continuous, normally distributed and showed equal variance. Analysis of the resultant pooled data between feeds was tested using a 2 way nested ANOVA. Unequal variances were found in the data and as such a Dunnetts T3 post hoc test was used to determine significance of the difference between feeds. Blood chemistry data were tested for normality and equality of variance, and a 2 way ANOVA was used to compare the effect of feed on blood chemistry parameters.

Castleman (2007) used a 2 way nested ANOVA to test if there were any significant differences between the morphological parameters in the mid intestine and distal intestine. Kolmogorov-Smirnov tests were used to assess the distribution of the data and Levene's Test of Equality of Error Variances, which showed that the data for these parameters did not have equal variance ($P<0.05$). However, as there is no non-parametric alternative to a 2 way nested ANOVA, the test was still used despite the assumption of equal variance being invalid. To overcome this, Dunnetts T3 post hoc tests were performed following any 2 way nested ANOVA on a data set that showed significant differences between feeds. Dunnetts T3 tests do not assume equal variance and as such valid significant differences between feeds can be observed from their use.

Particle size distributions were first analysed for their Chi-Square statistic to determine if H_0, that the observed frequencies were homogenous, could be accepted before further analysis was carried out. The skewness and kurtosis of the distributions were then determined. In this application, the determination of skewness is the more important of the two parameters as this can allow us to

compare and contrast between distributions resulting from faeces produced by fish consuming different feeds. A greater absolute value of skewness shows a more positively skewed distribution (a normal Gaussian distribution has a skewness of 0). Graphically this would be represented by a longer right tail to the distribution plot and a greater mass of the distribution to the left of the plot. Therefore in this use of the skew analysis, a distribution demonstrating a positive skewness value would contain a greater abundance of finer particles. A value closer to 0 would demonstrate a more equal distribution of coarse to fine particles. In terms of kurtosis, values of less than 3 would suggest that no one part of the particle size range dominates in any plot and the plot is referred to as being platykurtic (flat). This is what would be expected from the distributions found in the method development stage.

Once these values were calculated for each replicate, normality of the entire dataset (three data points per feed) was tested as above, and in the case of a normally distributed or successfully transformed data set, significance was tested for using a 2 way ANOVA and Duncan post hoc test. The two parameters were then plotted against each other as an x-y scatter plot and regressed, allowing any relationship between kurtosis and skewness to be determined. Regression was also carried out with ζ and kurtosis and skewness to determine whether there was any relationship between ζ and particle size.

In order to get an appreciation of the differences between different particle settling profiles, the time taken for 50% and 90% of the distribution to settle in 10 cm of water was determined as per Chapter 2, Section 2.16, and tested statistically, as for any of the other data sets containing three replicates per feed.

Rheological data were tested by determining the difference between the linear relationships resulting from plotting $\log_{10}$ frequency (Hz) against $\log_{10}$ complex viscosity (Pa.S). This was carried out using an ANCOVA on the $\log_{10}$ complex viscosity data (main effect) with "tank" as the covariate and incorporating a Levene's Test of Equality of Error Variances.

Chapter 3

Design and evaluation of feeds for Atlantic cod (*Gadus morhua*) and European sea bass (*Dicentrarchus labrax*) with fish meal substituted by plant proteins (Phase 1)

3.1 Introduction and objectives

The need to reduce the reliance of the aquaculture industry on wild caught fish meal while also limiting the effluent discharge and limiting the environmental effects of those discharges are pressing concerns within the industry. While moving forward with the substitution of fish meal in aquaculture feeds, it is necessary to ensure that 'new' protein sources result in comparable fish performance to traditional fish meal-based feeds. Researchers have therefore studied a diversity of plant protein sources for numerous fish species, as discussed in Chapter 1, Section 1.1. In order to assess the effectiveness of plant proteins, it is necessary to measure the following basic parameters.

The most basic measurement is fish weight change over the course of a feeding experiment. Within the published literature, biomass change is typically recorded every 2 to 6 weeks, depending on the length of the study, most often by bulk weighing the entire population in each rearing tank. In addition, weight specific growth rate (SGR), ratio of feed consumed to weight gained (feed conversion ratio; FCR), and changes in whole body composition (proximate analysis) are also widely measured parameters.

In addition to growth and feed intake-related parameters, it is important to record feed digestibility, to ensure that the nutrients within the experimental feeds are readily available. Most authors carry out the growth and digestibility sections of the experiments separately, or only focus on either growth or digestibility. However, some authors have used the same fish populations to investigate both parameters , by collecting faeces at the end of a growth experiment (Choi *et al.*, 2004). In many digestibility experiments, samples were taken once a day in the morning (Regost *et al.*, 1999, Pereira and Oliva-Teles, 2002) or 15 hr after feeding, in the morning (Glencross and Hawkins, 2004), to ensure that feed from the previous day has been fully processed prior to collection of faeces. From digestibility data, it is also possible to determine the amount of faeces that will be produced on any particular feed, which is of great importance when one is interested in the effects of protein substitution on the quantity and physical properties of effluents.

There are several ways in which faeces can be collected in an experimental system, and there are limitations with each. Collection of faeces in settling columns can result in overestimation of digestibility due to leaching of dry matter and nutrients, although rapid settlement of faeces can reduce this problem. If the settling is rapid (20 – 30 s) then standing time of anywhere between 2 and 18 hr will result in minimal nutrient leaching if held in ice (Fernandez *et al.*, 1998, Allan *et al.*, 1999). Dissection of faeces from euthanised animals and the physical stripping of faeces from live animals are very similar in their determination of digestibility (Storebakken *et al.*, 1998, Hemre *et al.*, 2003). There are also limitations with stripping, with reliability potentially being compromised due to incomplete digestion and inter operator differences in technique/position of start in intestinal tract (Weatherup and McCracken, 1998, Allan *et al.*, 1999). This method is however believed to be more suitable for novel protein assessments as the digestibility data obtained are inherently conservative (Glencross *et al.*, 2005).

The settling columns fitted to the tank outlets in the current experiment were unsuitable for collecting small volumes of faeces, and prolonged exposure to water could be expected to result in changes in physical characteristics (see below). For these reasons, the faecal stripping approach was used. In terms of the mechanics of faecal stripping, gentle pressure can be applied from the ventral fin to the anal region (Aksnes and Opstvedt, 1998) or from the pelvic fins to the anus (Fernandez *et al.*, 1996), depending on the length of the hindgut in the species of interest. In a previous study with Atlantic cod, pressure was placed 10 cm anterior and at 45° angle to the anus opening, with fish of 39 cm in length (Hemre *et al.*, 2003). This method may need to be adjusted for smaller fish. Soft pressure near the anus before stripping is preferable to remove any urine that would otherwise affect the subsequent measurements (Fernandez *et al.*, 1996).

Direct stripping of faeces is also beneficial in minimising any effects of leaching, soaking and interparticle interactions in water. The latter is particularly important in this study, where the physical properties of the faeces are to be analysed in terms of particle size distribution, surface charges and quantity of faeces produced. This data will be applied to assess the suitability of different plant protein sources for use in

enclosed rearing systems versus cages, as the requirements for each are different (see Chapter 1).

The objectives of the Phase 1 experiments were therefore as follows:

1. Determine whether Atlantic cod and European sea bass can utilise a variety of different plant protein sources at a fixed fish meal substitution rate (25% inclusion by weight);

2. Validate methods for measuring the physical properties of fish faeces;

3. Investigate whether the inclusion of different plant protein sources influences the physical properties of fish faeces and consider any downstream effects on aquaculture water quality control / environmental impact.

3.2 Experimental design

3.2.1 Feeds

A series of feeds (Table 3) were designed and manufactured according to the method described in Chapter 2, Section 2.4, to supply the nutritional requirements of Atlantic cod and European sea bass. They consisted of fish meal, and five feeds in which 25% by weight was made up of one of five different plant protein sources, these being high protein soybean meal (HiPro soya), low protein soybean meal (LoPro soya), soy protein concentrate (soy conc.), wheat gluten and lupin meal. A further set of feeds were formulated and manufactured to include whole faba beans and dehulled faba beans, again at a 25% inclusion. As these feeds were manufactured using different batches of raw materials from the first production of feeds, a second fish meal was also produced at this time to enable a direct comparison in the case of differences being observed.

Table 3: Feed formulations for Phase 1 feeds for Atlantic cod and European sea bass, produced in October 2005 and November 2006. All ingredients reported as g kg^{-1}. Feeds in bold were produced exclusively for the European sea bass experiment.

	Fish meal (control) 1	**Fish meal (control) 2**	HiPro soya	LoPro soya	Wheat gluten	Lupin meal	Soy conc.	**Whole faba beans**	**Dehulled faba beans**
Fish meal	732	**757**	563	540	468	594	505	**666**	**664**
Fish oil	98	**75**	111	108	107	91	116	**80**	**80**
HiPro soya (48% protein)	-	-	250	-	-	-	-	-	-
LoPro soya (46% protein)	-	-	-	250	-	-	-	-	-
Soy conc.	-	-	-	-	-	-	250	-	-
Wheat gluten	-	-	-	-	250	-	-	-	-
Lupin seed meal	-	-	-	-	-	250	-	-	-
Whole faba bean meal	-	-	-	-	-	-	-	**250**	-
Dehulled faba bean meal	-	-	-	-	-	-	-	-	**250**
Mineral and vitamin premix	3	**3**	3	3	3	3	3	**3**	**3**
Yttrium oxide premix	1	**1**	1	1	1	1	1	**1**	**1**
Wheat starch	166	**164**	72	99	171	61	125	**0**	**2**

All feeds were designed to be nutritionally balanced, and their proximate compositions are shown in Table 4.

Table 4: Proximate composition of Phase 1 feeds for Atlantic cod and European sea bass, g kg^{-1} feed as fed, determined as per Chapter 2, Section 2.11. Feeds in bold were produced exclusively for the European sea bass experiment.

	Fish meal (control) 1	**Fish meal (control) 2**	HiPro soya	LoPro soya	Wheat gluten	Lupin meal	Soy conc.	**Whole faba beans**	**Dehulled faba beans**
Fat	171	**165**	170	168	155	169	168	**160**	**160**
Protein	506	**548**	519	488	535	532	527	**526**	**526**
Ash	110	**100**	85	102	70	85	100	**92**	**92**
Dry matter	903	**944**	916	938	921	931	919	**925**	**925**

3.2.2 Rearing system

As described in Chapter 2, Section 2.1.

3.2.3 Source of animals

Atlantic cod were supplied by Manx Mariculture Ltd (Isle of Man) and were maintained in quarantine from 30th July 2005 to 12th September 2005, with a survival rate of 98.4%.

European sea bass were supplied by Aquastream (France) and were maintained in quarantine from 11th July 2006 to 4th October 2006, with a survival rate of 97.7%.

3.2.4 Feeding and sampling regime

3.2.4.1 ***Atlantic cod.*** Five days prior to the beginning of the study, 15 juvenile cod were randomly assigned to each of 18 tanks (i.e. 3 tank replicates for each of 6 feeds). The source population of cod had been size-sorted in advance and the large and small fish discarded. The selected fish were allowed to acclimate to their new tanks, being fed a mixture of 2.2 mm and 3.4 mm Skretting Europa© 15 pellets, supplied by Skretting.

On day 0 (22nd February 2006) an initial sample of 18 cod (1 from each tank) were euthanised according to the method described in Chapter 2, Section 2.6, and measurements made of whole body wet weight (80.23 g ± 9.92, mean ± SD) and

excised liver wet weight (6.62 g ± 1.91; HSI 8.13% ± 1.50, mean ± SD). Each fish was bled and dissected samples of GI tract (mid intestine and distal intestine) were dissected out and preserved in Bouin's solution. The mid intestine was classified as the section of the GI tract distal to the pyloric caecae and proximal to the increase in gut width. The distal intestine was classified as the section distal to the end of the mid intestine and proximal to the anus.

The experimental fish were fed to excess using a single 12 hr timed belt feeder per tank for the duration of the experiment. Skretting 3 mm experiment pellets were fed to the fish up to 90 g individual fish weight following which pellet size was increased to 4 mm. Water temperature was programmed to 13°C, at a salinity of 30 ppt and a pH of 8.0 (range 7.9 to 8.2). Lighting was by halogen lamps programmed to a 12 hr light/ 12 hr dark cycle, and set to replicate dawn and dusk by brightening and darkening gradually over a period of 15 min. Light intensity was 73 lux at the water's surface, averaged across all tanks.
Feed consumption was determined using a timed feeding period of a known quantity of feed and subsequent collection of uneaten pellets. Throughout the experiment, feed intake was recorded in this manner every three weeks, as was batch weight, whereby all fish in a tank were weighed together to determine total biomass.

Termination of the experiment and collection of final data began on day 112, 14th June 2006. All fish were euthanised in tank order, their weight was recorded and any notes on appearance or visible pathology were recorded. From the first 6 fish randomly selected from each tank, a blood sample was also taken from the caudal artery, the liver was excised and weighed, and samples of GI tract were taken as above. These dissected fish were not stripped for faeces so as to prevent damage to the GI tract. The remaining 7 to 9 fish from each tank were stripped of their faeces for analysis and their carcasses discarded.

3.2.4.2 ***European sea bass.*** One week prior to the beginning of the study, 29 juvenile sea bass were randomly assigned to each of 29 tanks (i.e., 3 tank replicates for each of 9 experimental feeds, plus 2 tank replicates for commercial control feed). The source population of sea bass had been size-sorted in advance and the large and small

fish discarded. The fish were allowed to acclimate to their new tanks, being fed 3.4 mm Skretting Europa© 15 pellets, supplied by Skretting.

On day 0 (17th January, 2007), an initial sample of 29 sea bass (1 from each tank) were euthanised and weighed (47.30 ± 7.16 g, mean ± SD, n = 29). Blood samples were taken from the ventral caudal vein and of 16 of these fish, which were also dissected for measurement of liver wet weight (0.86 g ± 0.23; HSI 1.82% ± 0.42, mean ± SD) and collection of GI tract samples (as per Chapter 3, Section 3.2.4.1). The remaining 13 fish were frozen whole for analysis of day 0 whole body proximate composition.

The sea bass were fed by hand to apparent satiation for the duration of the growth experiment. Skretting 3 mm experiment pellets were fed to the fish up to 90 g individual body weight following which pellet size was increased to 4 mm. Those fish receiving the commercial control feed were fed the 3.4 mm Skretting Europa© 15 pellet up to 120 g individual fish weight, followed by the 5.4 mm Skretting Europa© 18 pellet. Water temperature was programmed to 24°C (min 23.3°C, max 25.3°C), at a salinity of 30 ppt and a pH of 8.0 (range 7.9 to 8.2). Lighting was as per the preceding cod experiment (see Chapter 3, Section 3.2.4.1).

Feed intake was measured and recorded twice weekly, for calculation of mean daily feed consumption. Each tank was fed from a pot containing a known weight of feed, which was weighed twice a week to calculate daily feed consumption. The fish in each tank were also batch weighed every two weeks. The combined feed consumption and fish weight data were used to determine feed intake, SGR and FCR.

The sea bass growth experiment was terminated on day 97, 24th April 2007, at which point all fish were anaesthetised for measurement of individual weight. 6 fish per tank were euthanised and samples of liver and GI tract (see Chapter 3, Section 3.2.4.1) were collected and preserved in Bouin's solution. The carcasses of these fish, including the remains of the liver and GI tract, were then bagged and frozen immediately for analysis of whole body proximate composition as per Chapter 2, Section 2.11. All remaining sea bass were allowed to recover from anaesthesia and returned to their tanks for the feed digestibility phase of the experiment.

For measurement of feed digestibility, the sea bass were fed using a single 12 hr clockwork belt feeder per tank, rather than feeding by hand. Lighting was programmed to a 15.5 hr light/ 8.5 hr dark cycle, with the fish being fed one meal per day for an hour before sunset, to ensure that faeces were stripped during the peak of faecal production following feeding i.e. approximately 9.0 to 10.5 hr after feeding (Dias *et al.*, 1998).

At simulated dawn on the day of stripping, all fish were netted from their holding tanks and anaesthetised to the point of loss of balance. They were then removed from the anaesthetic by hand with the use of a cloth, held over the head of the fish to reduce stress. Urine was first expelled by placing a gentle pressure slightly anterior to the vent. Fish were then stripped of faeces by placing a light pressure on the abdomen slightly anterior of the vent using forefinger and thumb, and then by making movements towards the posterior of the fish. The faeces were dispensed into a 60 ml sterilin pot and pooled per tank. The fish were then returned to their holding tank where their recovery was observed. This was repeated twice per week until sufficient quantity of sea bass faeces was collected from each tank replicate.

Faeces from all stripping events over a period of approximately 12 weeks were pooled and kept frozen at -30°C pending sub sampling for subsequent analysis.

3.2.5 Sample analyses

All sample analyses, data handling and statistical analyses were carried out in accordance with the methods detailed in Chapter 2, General Materials and Method Development.

3.3 Results

3.3.1 Atlantic cod

3.3.1.1 ***Survival, growth and feed utilisation.*** The mean cumulative survival rate of Atlantic cod across all feed groups was 96.8% with no statistically significant differences observed among diets. Final body weight, body weight increase, SGR, FCR and feed intake data are summarised in Table 5. It was noted before the experiment that the lupin meal feed was much less stable in water than any of the

other feeds. For this reason, the feed intake data for the lupin meal feed, calculated from retrieved pellets was deemed to be inaccurate therefore the feed intake and FCR data for this feed were omitted from statistical analysis. Data for each of the parameters were determined to be normally distributed; statistically significant differences were found among feed groups for final body weight, body weight increase, and SGR. Mean final body weight ranged from 214.45 to 256.55 g, body weight increase from 1.38 to 1.82 g day^{-1}, SGR from 0.88 to 1.04% b. wt. day^{-1}, FCR from 0.71 to 1.07 and feed intake from 0.64 to 0.97%. Mean final body weight, SGR and body weight increase were significantly greater for the lupin meal feed than for fish meal, LoPro soya, soy conc., and wheat gluten feeds, with HiPro soya also being significantly greater than soy conc. Hepatosomatic index (HSI) data were also found to be normally distributed, with homogeneous variance and no violation of the homogeneity of regression slopes. No statistically significant differences in HSI were observed among feed groups, with an overall mean value of 9.76%.

Digestibility data based on analyses of single faecal samples are summarised in Table 6. Statistical analysis was not performed due to the lack of replication, and therefore the orders of digestibility, from most digestible to least digestible, are shown.

Dry matter: Fish meal > wheat gluten > soy conc. > LoPro soya > lupin meal > HiPro soya

Organic matter: Fish meal > wheat gluten > soy conc. > LoPro soya > lupin meal > HiPro soya

Protein: Wheat gluten > soy conc. > fish meal > lupin meal > LoPro soya > HiPro soya

Table 5: Fish growth, feed utilisation and hepatosomatic index data for Atlantic cod fed Phase 1 experimental feeds for 112 days. Values are mean ± standard deviation. Different superscript letters signify statistically significant differences among feed groups ($p < 0.05$). † Determined from start population representative biomass (= "batch weight"); • Determined from total tank biomass (= "batch weight") for each of three replicate tanks. ▲ $n = 18$ from individual weighing (6 fish per tank).

	Fish meal	HiPro soya	LoPro soya	Wheat gluten	Lupin meal	Soy conc.
Initial body weight (g)†	80.23 ± 9.92	80.23 ± 9.92	80.23 ± 9.92	80.23 ± 9.92	80.23 ± 9.92	80.23 ± 9.92
Final body weight (g)•	220.23 ± 65.66^{bc}	249.41 ± 50.40^{ab}	217.76 ± 54.78^{bc}	218.36 ± 68.71^{bc}	256.55 ± 68.95^{a}	214.45 ± 57.04^{c}
Body weight increase (g day^{-1})•	1.44 ± 0.25^{bc}	1.74 ± 0.11^{ab}	1.41 ± 0.21^{bc}	1.42 ± 0.13^{bc}	1.82 ± 0.14^{a}	1.38 ± 0.17^{c}
Specific growth rate (% b.wt. day^{-1})•	0.90 ± 0.10^{bc}	1.01 ± 0.04^{ab}	0.89 ± 0.08^{bc}	0.89 ± 0.05^{bc}	1.04 ± 0.05^{a}	0.88 ± 0.07^{c}
Feed conversion ratio•	0.71 ± 0.24	0.80 ± 0.05	0.98 ± 0.24	0.78 ± 0.22	ND$^{\Delta}$	1.07 ± 0.21
Feed intake (% b.wt. day^{-1})•	0.64 ± 0.15	0.85 ±0.04	0.89 ± 0.13	0.72 ± 0.17	ND$^{\Delta}$	0.97 ± 0.18
Hepatosomatic index▲	9.35 ± 2.65	10.78 ± 3.86	9.07 ± 2.76	9.94 ± 2.48	10.02 ± 2.49	9.42 ± 2.73

Table 6: Apparent digestibility of Phase 1 experimental feeds (dry matter, organic matter and protein) for Atlantic cod.

	Dry matter %	Organic matter %	Protein %
Fish meal	79.8	85.4	87.0
HiPro soya	68.1	74.6	83.4
LoPro soya	73.7	78.3	85.0
Wheat Gluten	75.5	81.4	89.6
Lupin meal	70.4	75.3	86.9
Soy conc.	75.2	79.4	88.2

3.3.1.2 ***Gross pathology, gastrointestinal morphometrics & blood chemistry.*** No signs of enteritis, as characterised by Baeverford and Krogdahl (1996), were observed in any samples of Atlantic cod mid intestine or distal intestine. Distal intestine was not analysed for morphometrics due to the amount of variation in the samples, attributed to faecal distension of the gut samples. Feed type had a significant effect on the proportional thickness of muscle and villus width in relation to the diameter of the mid intestine: those cod offered the lupin meal feed had a significantly reduced mid intestine muscle thickness than cod receiving the HiPro soya, LoPro soya or wheat gluten feeds. Additionally, HiPro soya-fed fish had significantly narrower villi than fish meal and wheat gluten. No statistically significant differences were observed in blood chemistry parameters among feeds.

A high incidence of gross abnormalities was observed across all feed groups, including a subopercular mass, thought to be thyroid hyperplasic tissue (Fig. 14), in 45.7% of cod at final sampling (n = 244). Another wide ranging abnormality observed was a bend in the body immediately posterior to the head or the ''stargazer'' syndrome (Fig. 14). Neither of these abnormalities was attributable to feed type (Kruskal-Wallace, $p > 0.05$) and it appears that these did not impair the growth performance of the experimental animals.

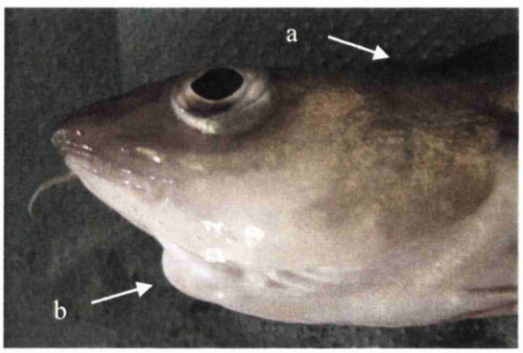

Figure 14: Photograph demonstrating the main abnormalities observed in the Phase 1 stock of cod. Note the upwards tilt of the head with the eyes looking skywards, commonly referred to as 'stargazing' (a), and the clearly visible subopercular mass (b).

3.3.1.3 *Faeces*

Particle size distribution

Faecal particle size distributions are shown in Figure 15, with all observed frequencies being homogeneous ($p < 0.01$). Three main peaks were observed, with the second two being common among all feeds at approximately 210 and 480 µm. There was a notable shift in the first peak between those of fish meal and wheat gluten which occurred at approximately 30 µm, lupin meal which occurred at approximately 70 µm, and all other feeds which occurred at approximately 120 µm.

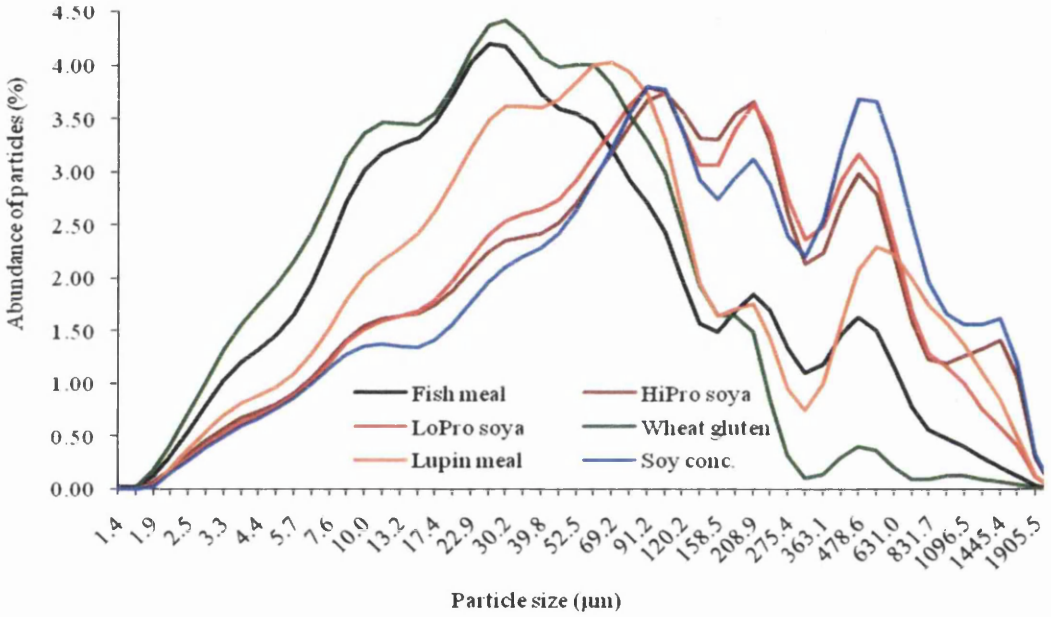

Figure 15: Faecal particle size distributions produced by Atlantic cod fed Phase 1 experimental feeds.

Results for particle size distribution kurtosis and skewness for Atlantic cod faeces are summarised in Figure 16. The kurtosis dataset was found to be normally distributed and demonstrated statistically significant differences among feeds after parametric analysis. The skewness dataset was normally distributed and parametric analysis showed that no statistically significant differences were present.

All feeds demonstrated highly platykurtic plots (negative values, relatively flat plots), suggesting little dominance of any one particle size range. Fish meal and wheat gluten feeds resulted in plots which were significantly more platykurtic (-1.41 and -1.45 respectively) than those for lupin meal, HiPro soya and soy conc. (-1.07, -

0.95 and -0.97 respectively). Kurtosis plots for HiPro soya and soy conc. were also significantly less platykurtic than LoPro soya (-1.31) and lupin meal.

Regression of kurtosis against skewness (Fig. 17) showed that there was no statistically significant relationship between these two parameters for Atlantic cod faecal particles ($F_{1, 19} = 3.089$, $r^2 = 0.095$, $p > 0.05$).

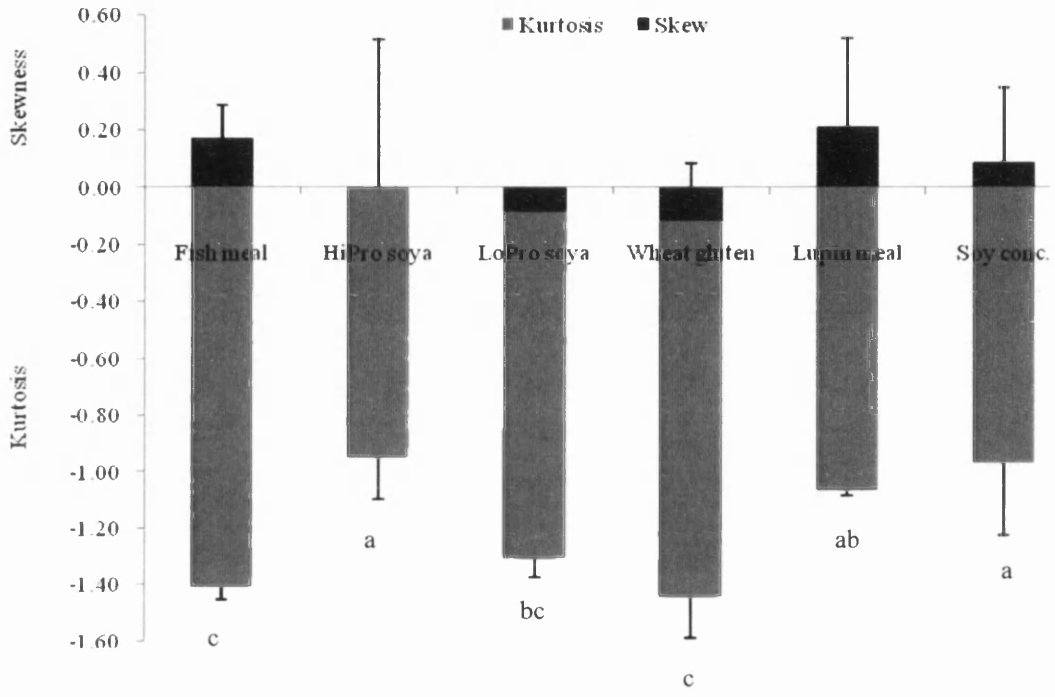

Figure 16: Kurtosis and skewness of faeces particle size distributions derived from the faeces of Atlantic cod fed Phase 1 experimental feeds. Values are mean ± standard deviation. Statistically significant differences are denoted by different superscript letters ($p < 0.05$).

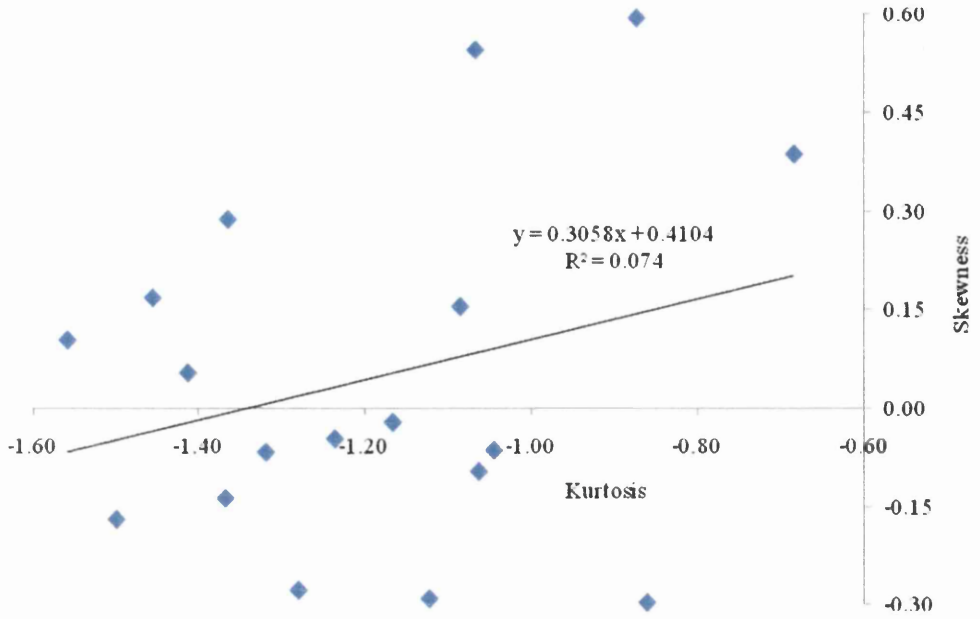

Figure 17: Scatter plot and linear regression of faecal particle size distribution kurtosis against skewness to determine any relationships between the two parameters in the case of Atlantic cod receiving Phase 1 experimental feeds.

Calculated faecal production

Faecal production (Fig. 18) was determined for all feeds as described in Chapter 2, Section 2.15. These data were derived from a single replicate of digestibility per feed, i.e. one tank per feed, due to a lack of material from some tanks. This single datum per feed had to therefore be taken as being representative of the feed and was used to calculated faecal production for each replicate. Data for the production of faeces in terms of g kg^{-1} growth and production in the "up to 100 µm" and "up to 240 µm" particle size ranges were normally distributed. Total faecal production was 132.0, 235.1, 237.0, 176.1 and 243.0 g kg^{-1} for fish meal, HiPro soya, LoPro soya, wheat gluten and soy conc. respectively. No statistically significant differences were noted at any range.

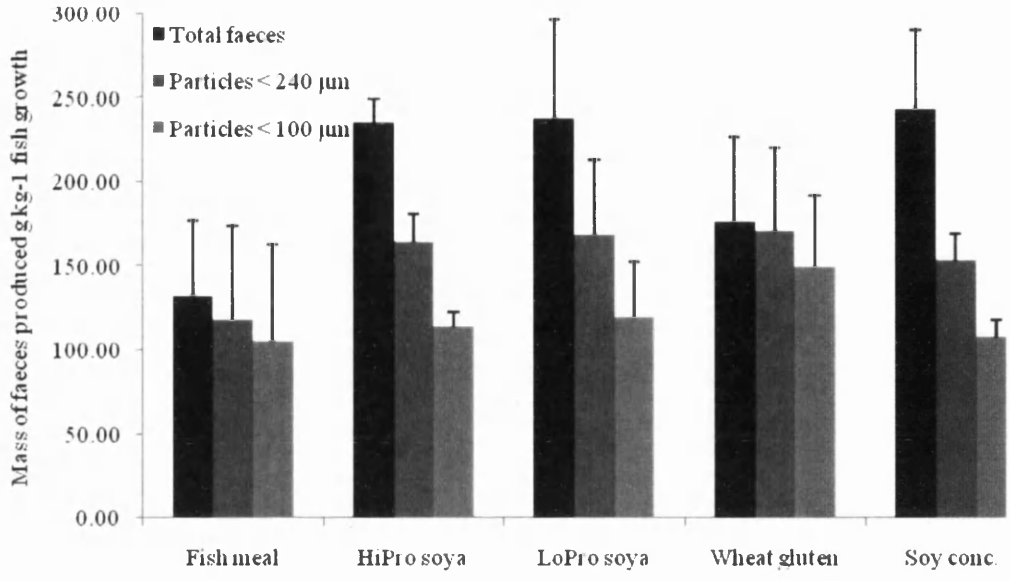

Figure 18: Mass of total faeces, faecal particles below 100 µm and faecal particles below 240 µm produced g kg^{-1} growth for Atlantic cod receiving Phase 1 experimental feeds. Values are mean ± standard deviation. No significant differences were observed ($p > 0.05$). The data for the lupin meal feed is omitted due to concerns with the accuracy of the feed intake and resulting FCR data for this feed.

Settling characteristics of faecal material

The mean settling velocity of Atlantic cod faecal particles calculated from particle size and density was 4.0 cm s^{-1} (overall mean for all replicates from all Phase 1 feeds, as no statistically significant differences were found among feeds). This value was used to determine the time taken for faecal particles to settle in 10 cm of water, as detailed in Chapter 2, Section 2.16.

The settlement data are presented in two plots, split to allow better resolution at the lower end of the x axis, the first (Fig. 19) representing 10 to 70% of the distribution, and the second (Fig. 20) representing 70 to 95% of the distribution. The data for the 0 to 10% fraction of the particle size distribution are excluded from the dataset due to this being made up of the coarsest particles in the distribution, which would be expected to settle out of suspension almost instantaneously. The 95 to 100% fraction of the distribution was also excluded, since these finest particles would be expected to remain in suspension indefinitely. The data for settlement time of faecal particles

at both 50% and 90% fraction of distribution were found to be non-normally distributed, and were tested non-parametrically as no transformation was possible.

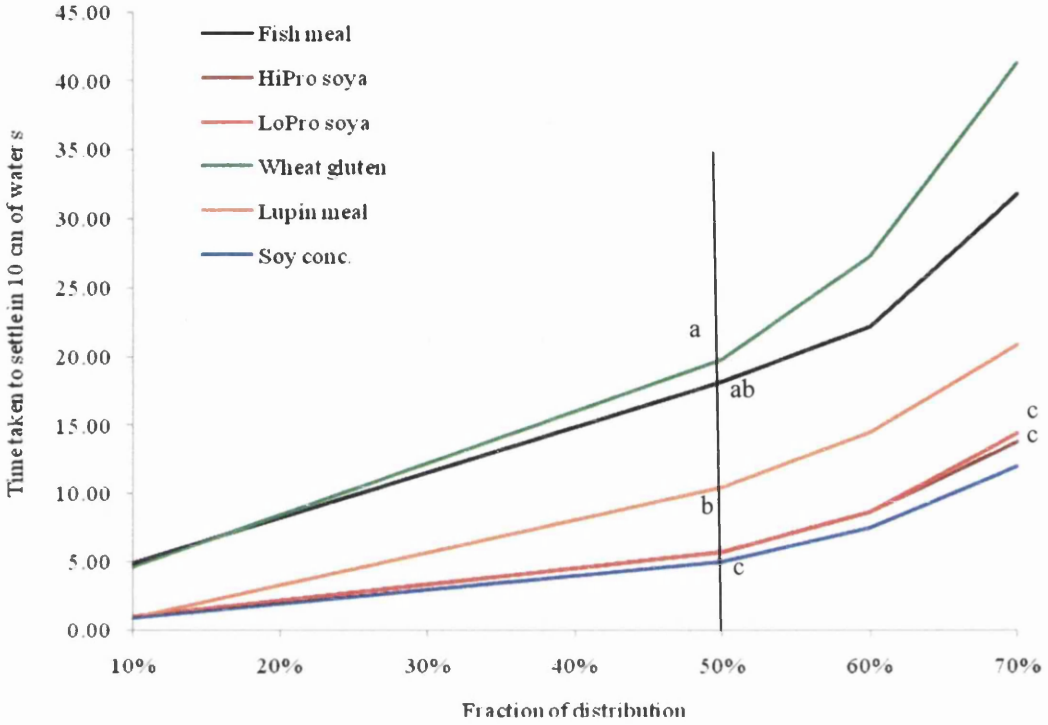

Figure 19: Calculated time taken (s) for Atlantic cod faecal particles in the range 10 to 70% of the total particle size distribution (from coarse to fine particles) to settle in 10 cm of water. Different letters signify statistically significant differences in the time taken for 50% of the distribution to settle ($p < 0.05$).

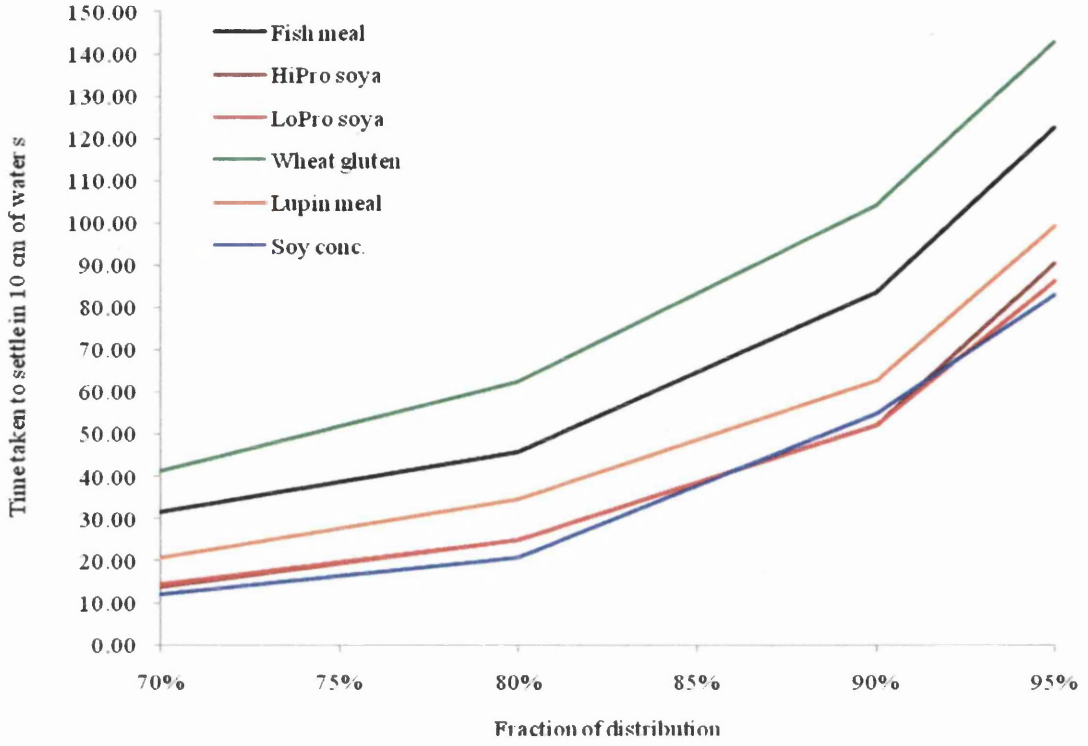

Figure 20: Calculated time taken (s) for Atlantic cod faecal particles in the range 70 to 90% of the total particle size distribution (from coarse to fine particles) to settle in 10 cm of water. No statistically significant differences were noted in the time taken for 90% of the distribution to settle ($p > 0.05$).

Statistically significant differences in faeces settlement time were observed among feeds at the 50% fraction of the distribution. Faeces from the wheat gluten feed yielded faeces with significantly slower settling rates than HiPro soya, LoPro soya, soy conc. and lupin meal. The lupin meal feed in turn yielded faeces with significantly slower settling rates than HiPro soya, LoPro soya and soy conc., the latter exhibiting the most rapid faeces settlement of all the tested feeds.

3.3.2 European sea bass

3.3.2.1 *Survival, growth, feed utilisation and whole body proximate composition.*

The mean cumulative survival rate of European sea bass across all feed groups was 99.9% with no statistically significant differences observed among diets. No significant differences were observed for any parameter between the two fish meal feeds (fish meal 1 and fish meal 2); therefore only fish meal 1 was included in the statistical comparisons with experimental feeds. Final body weight, body weight increase, SGR, FCR and feed intake data for sea bass are summarised in Table 8. Data for each of these parameters were determined to be normally distributed; no

statistically significant differences were found among feed groups for any of these parameters. Mean final body weight ranged from 133.00 to 143.61 g, body weight increase from 0.88 to 0.99 g day^{-1}, SGR from 1.06 to 1.14% b. wt. day^{-1}, FCR from 1.23 to 1.32 and feed intake from 1.37 to 1.56%. Hepatosomatic index was also found to be normally distributed, with homogenous variance and no violation of the homogeneity of regression slopes. Statistically significant differences in HSI were observed, with an overall mean value of 1.54%. However, the nested ANOVA and GLM III analyses carried out on the HSI data demonstrated that both feed and tank significantly affected the data independently, i.e. there were statistically significant differences observed among replicates within experimental feeds, as well as among feeds. The statistical significances of the differences in HSI among feeds were therefore deemed to be unsafe.

End point values for sea bass whole body proximate composition (as g kg^{-1} live weight) are summarised in Table 7. The lipid and ash datasets were normally distributed, and were tested parametrically. The protein content and dry matter datasets were non-normally distributed. The protein content data were successfully transformed and tested parametrically, while the dry matter data were untransformable and was therefore non-parametrically tested. No statistically significant differences were observed among feed groups for any of the proximate composition parameters.

Table 7: Proximate composition (g kg^{-1} live weight) of whole ground European sea bass carcasses after receiving Phase 1 experimental feeds for 97 days. Values are mean ± standard deviation (n = 3). No significant differences among diets were noted ($p > 0.05$).

	Fish meal	HiPro soya	LoPro soya	Wheat gluten	Lupin meal	Soy conc.	Whole faba beans	Dehulled faba beans
Protein g kg^{-1}	174 ± 4	179 ± 6	177 ± 3	176 ± 4	173 ± 9	175 ± 4	175 ± 6	181 ± 6
Lipid g kg^{-1}	157 ± 5	153 ± 7	156 ± 6	160 ±3	157 ± 14	174 ± 17	147 ± 9	156 ± 6
Ash g kg^{-1}	36 ± 2	39 ± 4	37 ± 2	37 ± 3	37 ± 1	36 ± 2	38 ± 3	37 ± 1
Dry matter g kg^{-1}	367 ± 13	374 ± 7	374 ± 4	376 ± 6	369 ± 19	386 ± 16	367 ± 9	374 ± 8

Table 8: Fish growth, feed utilisation and hepatosomatic index data for European sea bass fed Phase 1 experimental feeds for 97 days. Values are mean ± standard deviation. Different superscript letters signify statistically significant differences among feed groups ($p < 0.05$). † Determined from start population representative biomass (= "batch weight"); • Determined from total tank biomass (= "batch weight") for each of three replicate tanks. ▲ $n = 18$ from individual weighing (6 fish per tank).

	Fish meal	HiPro soya	LoPro soya	Wheat gluten	Lupin meal	Soy conc.	Whole faba beans	Dehulled faba beans
Initial body weight (g)†	47.30 ± 7.16	47.30 ± 7.16	47.30 ± 7.16	47.30 ± 7.16	47.30 ± 7.16	47.30 ± 7.16	47.30 ± 7.16	47.30 ± 7.16
Final body weight (g)•	135.19 ± 26.74	134.63 ± 28.94	143.61 ± 29.01	134.3 ± 35.18	136.51 ± 28.02	133.00 ± 28.68	141.48 ± 31.06	137.35 ± 28.68
Body weight increase (g day^{-1})•	0.91± 0.03	0.90 ± 0.15	0.99 ± 0.13	0.90 ± 0.22	0.92 ± 0.07	0.88 ± 0.09	0.97 ± 0.08	0.93 ± 0.04
Specific growth rate (% b.wt. day^{-1})•	1.08± 0.02	1.08 ± 0.11	1.14 ± 0.09	1.07 ± 0.18	1.09 ± 0.05	1.06 ± 0.07	1.13 ± 0.06	1.10 ± 0.03
Feed conversion ratio•	1.24 ± 0.07	1.27 ± 0.12	1.30 ± 0.09	1.27 ± 0.19	1.32 ± 0.12	1.24 ± 0.06	1.23 ± 0.07	1.23 ± 0.09
Feed intake (% b.wt. day^{-1})•	1.41 ± 0.05	1.42 ± 0.05	1.56 ± 0.04	1.39 ± 0.09	1.51 ± 0.08	1.37 ± 0.09	1.45 ± 0.06	1.41 ± 0.00
Hepatosomatic index▲	1.72 ± 0.08^{b}	1.47 ± 0.09^{cd}	1.25 ± 0.06^{d}	2.06 ± 0.12^{a}	1.39 ± 0.09^{cd}	1.56 ± 0.08^{bc}	1.45 ± 0.08^{cd}	1.38 ± 0.08^{cd}

Feed digestibility data are summarised in Table 9. Organic matter digestibility was not determined due to ash content data not being collected. Only two replicates were possible per feed, and as such 99% confidence intervals were determined. These were found to be large due to the low value of *n*, and as such the data were not analysed for statistical significance of differences.

Table 9: Apparent digestibility of Phase 1 experimental feeds (dry matter and protein) for European sea bass. Values are mean ± standard deviation ($n = 2$).

	Dry matter %	Protein %
Fish meal	79.2 ± 6.7	90.2 ± 2.0
HiPro soya	70.2 ± 3.2	87.8 ± 0.8
LoPro soya	69.0 ± 2.3	89.5 ± 0.9
Wheat gluten	82.9 ± 0.1	93.0 ± 0.8
Lupin meal	66.7 ± 2.3	89.6 ± 0.6
Soy conc.	72.9 ± 0.6	91.5 ± 0.4
Whole faba beans	68.6 ± 0.7	87.8 ± 1.3
Dehulled faba beans	75.7 ± 2.8	90.8 ± 0.0

The orders of digestibility, from most digestible to least digestible, were as follows.

Dry matter: Wheat gluten > fish meal > dehulled faba beans > soy conc. > HiPro soya > LoPro soya > whole faba beans > lupin meal

Protein: Wheat gluten > soy conc. > dehulled faba beans > fish meal > lupin meal > LoPro soya > whole faba beans = HiPro soya

3.3.2.2 ***Gastrointestinal morphometrics.*** No signs of enteritis, as characterised by Baeverford and Krogdahl (1996), were observed in any samples of sea bass mid intestine or distal intestine. Statistically significant differences were observed in villus diameter, which was significantly reduced among fish fed the lupin meal feed compared with fish meal and LoPro soya, and significantly reduced in soy conc. compared to fish meal. Villus surface area was also significantly reduced in fish fed dehulled faba beans over fish meal, whole faba beans, soy conc., lupin meal and HiPro soya. Finally, statistically significant differences were found in the diameter of the *lamina propria*. LoPro soya resulted in a significantly greater diameter than fish

meal, wheat gluten, soy conc., lupin meal, HiPro soya and dehulled faba beans, with whole faba beans resulting in a significant increase over fish meal. In terms of distal intestine morphometrics, statistically significant differences were found in muscle thickness, where the fish meal feed resulted in significantly greater relative thickness than soy conc. In the case of villus length, dehulled faba beans resulted in significantly longer villi than HiPro soya.

3.3.2.3 *Faeces.*

Particle size distribution

As shown in Figure 21, all Phase 1 feeds resulted in similar shaped faecal particle size distribution profiles for Atlantic cod, with all observed frequencies being homogeneous ($p < 0.01$). Four main peaks were observed, common among all feeds at approximately 105, 210, 480 and 1445 µm.

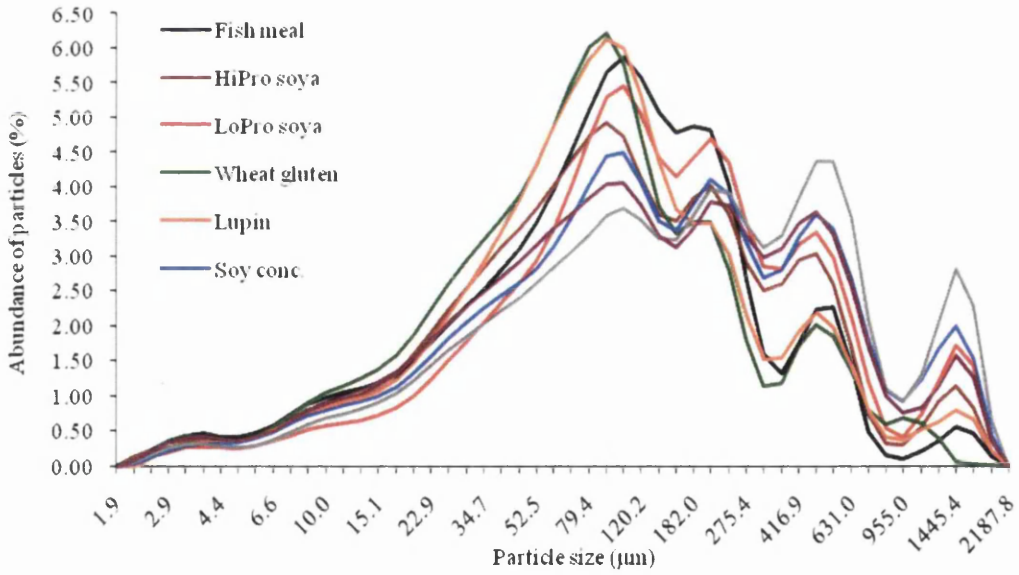

Figure 21: Faecal particle size distributions of faeces produced by European sea bass fed Phase 1 experimental feeds.

Results for particle size distribution kurtosis and skewness are summarised in Figure 22. The kurtosis dataset was found to be non-normally distributed, and was successfully transformed prior to being tested parametrically. The skewness dataset was normally distributed and, was parametrically tested. Both parameters showed statistically significant differences among feed groups.

All feeds demonstrated highly platykurtic plots (negative values, relatively flat plots), suggesting little dominance of any one particle size range. Whole faba beans, dehulled faba beans and soy conc. feeds resulted in plots which were significantly more platykurtic (-1.48, -1.40 and -1.45 respectively) than those resulting from LoPro soya, fish meal, wheat gluten and lupin meal (-1.04, -0.64, -0.26 and -0.22 respectively). Plots resulting from wheat gluten and lupin meal feeds were also significantly less platykurtic than those of LoPro soya and HiPro soya (-1.28). In terms of skewness, lupin meal and wheat gluten plots were significantly more positively skewed (0.97 and 0.88 respectively) than whole faba beans, dehulled faba beans, soy conc., HiPro soya and LoPro soya (0.05, 0.17, 0.17, 0.37 and 0.55 respectively). Whole faba beans also resulted in a significantly less skewed plot than HiPro soya.

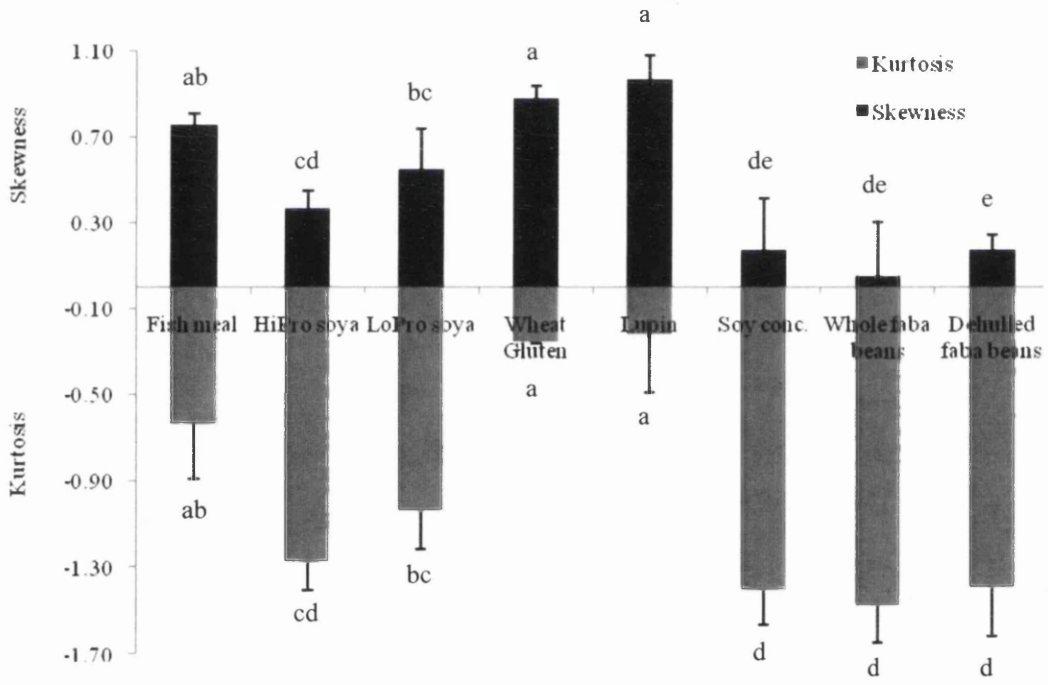

Figure 22: Kurtosis and skewness of particle size distributions derived from the faeces of European sea bass fed Phase 1 experimental feeds. Values are mean ± standard deviation. Different superscript letters signify statistically significant differences among feed groups ($p < 0.05$).

Regression of kurtosis against skewness (Fig. 23) revealed a highly statistically significant positive relationship between the two parameters for sea bass ($F_{1, 20} = 175.426$, $r^2 = 0.8979$, $p < 0.001$), demonstrating that the coarser the particles (lower value of skewness) the more platykurtic the distribution (lower value of kurtosis).

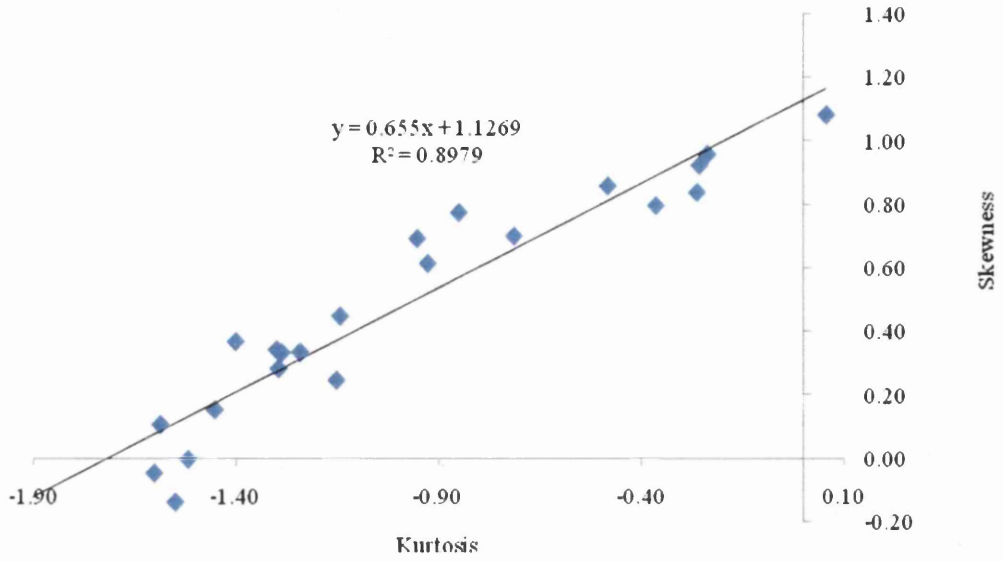

Figure 23: Scatter plot and linear regression of faecal particle size distribution kurtosis against skewness to determine any relationships between the two parameters in the case of European sea bass receiving Phase 1 experimental feeds.

Zeta potential of faecal particles

For completeness, the combined results of the 3 replicates of ζ from each feed and the mean of these results are shown in Figure 24. This shows that the ζ of the faeces is negative for the higher range of pH. A cross-over point is achieved at pH 1.95, the pH at zero point of charge (pH_{zpc}) or IEP of the material. The plot shows a relatively rapid decline in ζ between pH 2 and pH 7, followed by a slight increase in the ζ up to pH 9.8 and a subsequent drop at higher pH.

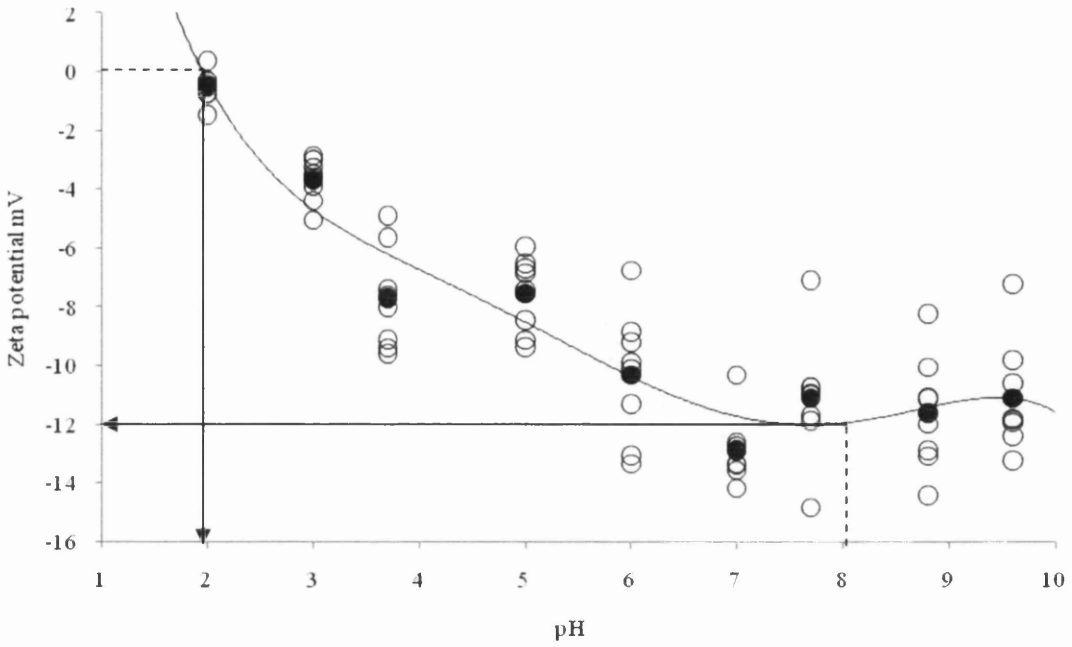

Figure 24: Combined zeta potential (ζ) plot of faeces from European sea bass fed Phase 1 experimental feeds. Trend line is fitted to the overall mean values, denoted by filled circles. The position of the combined zero point of charge (pH_{zpc}) or isoelectric point (IEP) is shown, and is calculated as pH 1.95. The ζ at pH 8.05 was calculated as -12.0 for the combined data.

ζ and IEP at the system operating pH of 8.05 are shown for each feed in Table 10. Data for both parameters were normally distributed, and no statistically significant differences were observed among feed groups for either parameter.

Table 10: Zeta (ζ) potential in millivolts (mV) at rearing system pH (pH 8.05) and isoelectric point (IEP) for faeces produced by European sea bass fed Phase 1 experimental feeds. Values are mean ± standard deviation. No statistically significant differences were observed ($p > 0.05$).

	ζ @ pH 8.05	IEP
Fish meal	-14.64 ± 4.38	1.81 ± 0.12
HiPro soya	-11.57 ± 3.17	1.94 ± 0.18
LoPro soya	-12.36 ± 6.03	1.99 ± 0.00
Wheat gluten	-11.10 ± 1.51	1.96 ± 0.03
Lupin meal	-8.45 ± 1.84	2.07 ± 0.04
Soy conc.	-13.43 ± 1.94	1.95 ± 0.06
Whole faba beans	-11.19 ± 2.41	1.97 ± 0.02
Dehulled faba beans	-11.52 ± 1.61	1.99 ± 0.09

Regression of ζ at pH 8.05 against the particle size distribution kurtosis showed that there was no statistically significant relationship between the parameters ($F_{1, 19}$ =

0.542, $r^2 = 0.028$, $p > 0.05$) or between ζ and particle size distribution skewness ($F_{1, 19} = 0.168$, $r^2 = 0.009$, $p > 0.05$).

Calculated faecal production

Faecal production (Fig. 25) was determined as described in Chapter 2, Section 2.15. Only two replicates were available, and 99% confidence intervals of the dataset were large due to the low value of *n*. The data were therefore not statistically analysed.

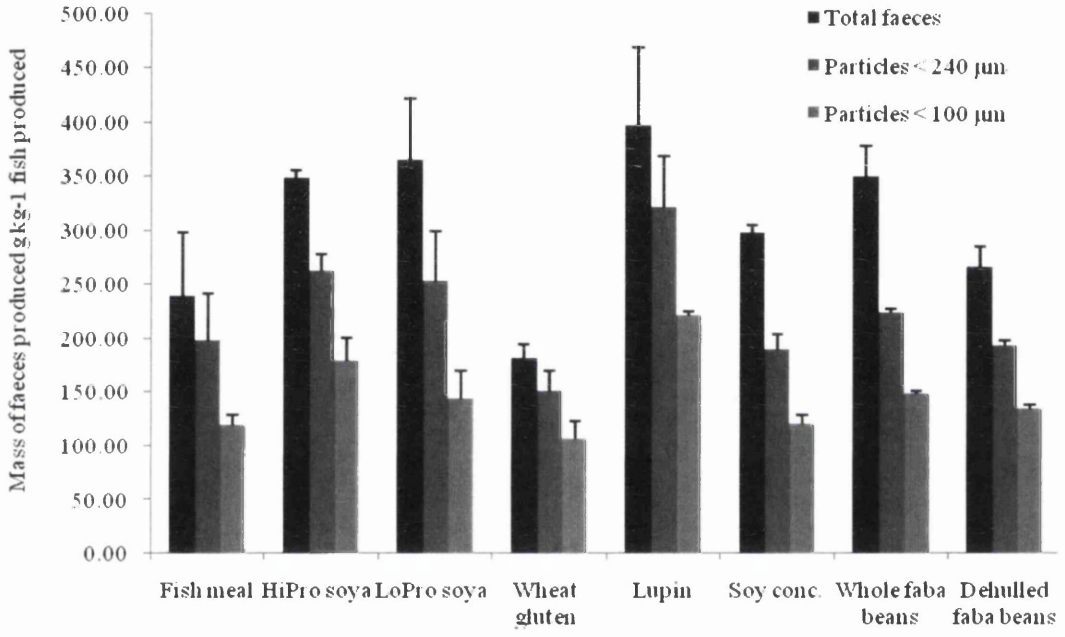

Figure 25: Mass of total faeces, faecal particles below 100 µm and faecal particles below 240 µm produced (g kg^{-1} growth) by European sea bass receiving Phase 1 experimental feeds. Values are mean ± standard deviation. No significant differences were noted ($p > 0.05$).

Faecal production ranged from 186 to 388 g (fish meal and whole faba beans). In the range of particles up to 240 µm, faecal production ranged from 156 to 288 g (fish meal and wheat gluten) and in the range up to 100 µm ranged from 95 to 203 g (fish meal and wheat gluten).

Settling characteristics of faecal particles

The mean settling velocity of European sea bass faecal particles calculated from particle size and density was 4.2 cm s^{-1} (overall mean for all replicates from all Phase

2 feeds, as no statistically significant differences were found among feeds). This value was used to determine the time taken for faeces to settle in 10 cm of water, as detailed in Chapter 2, Section 2.16.

The faeces settlement data are presented in two plots, split to allow better resolution at the lower end of the x axis, the first (Fig. 26) representing 10 to 70% of the distribution, and the second (Fig. 27) being 70 to 95% of the distribution. The data for the 0 to 10% fraction of the particle size distribution is excluded from the dataset due to this being made up of the coarsest particles in the distribution, which would be expected to settle out of suspension almost instantaneously. The 95 to 100% fraction of the distribution was also excluded, since these finest particles would be expected to remain in suspension indefinitely. The data for faeces settlement time at both the 50% and 90% fraction of distribution were found to be normally distributed, and were tested parametrically. No statistically significant differences were found among feed groups.

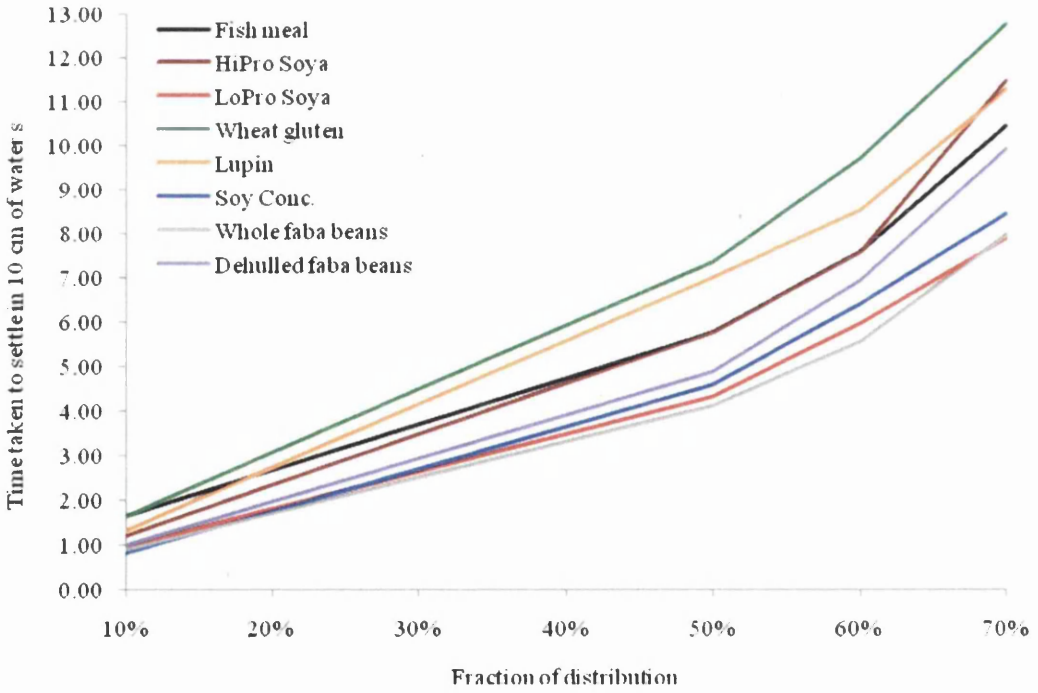

Figure 26: Calculated time taken (s) for European sea bass faecal particles in the range 10 to 70% of the total particle size distribution (from coarse to fine particles) to settle in 10 cm of water. No statistically significant differences were noted in the time taken for 50% of the distribution to settle ($p > 0.05$).

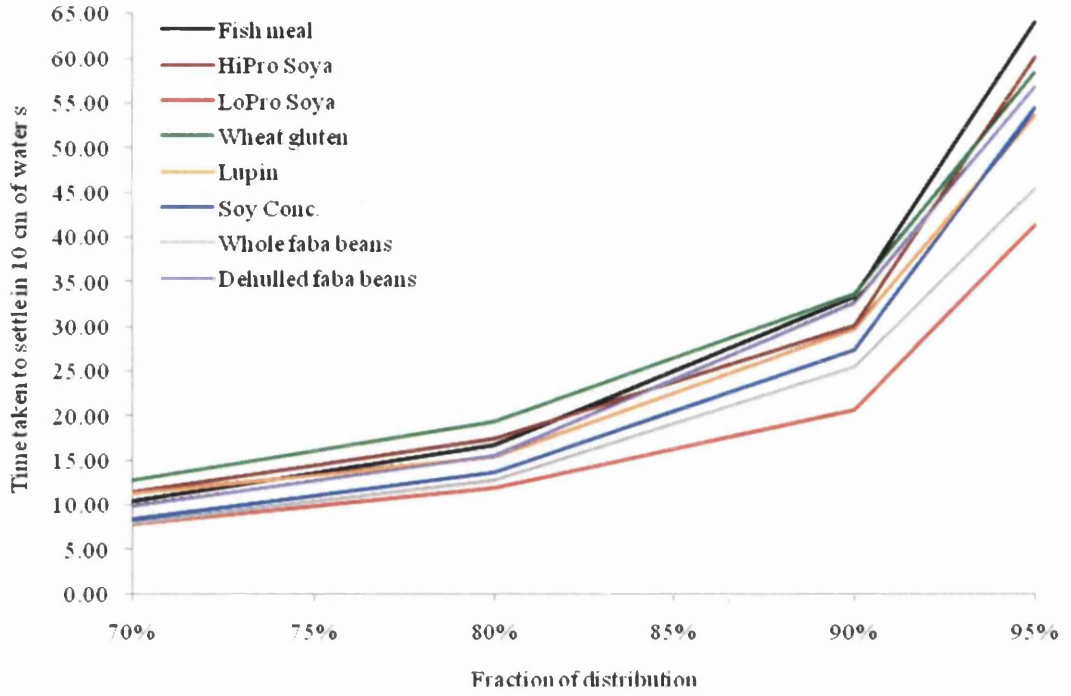

Figure 27: Calculated time taken (s) for European sea bass faecal particles in the range 70 to 90% of the total particle size distribution (from coarse to fine particles) to settle in 10 cm of water. No statistically significant differences were noted in the time taken for 90% of the distribution to settle ($p > 0.05$).

3.4 Discussion

Juvenile Atlantic cod and European sea bass consuming formulated feeds containing 25% by weight of one of 5 (cod) or 7 (sea bass) plant proteins in substitution for fish meal grew comparably to or better than those receiving the fish meal feed. The SGR and body weight increase of the cod were comparable with published values for fish of similar size under similar culture conditions at 0.94% and 1.54 g day^{-1} respectively, in comparison to the findings of Bjornsson and Olafsdottir (2006) at 0.97% and 1.40 g day^{-1} respectively. The sea bass performance was also in line with published data. Previous studies have also recorded positive results for these plant protein types for other marine and freshwater fish species, at a range of inclusion levels (Berge *et al.*, 1999, Day and Gonzalez, 2000, Kissil and Lupatsch, 2004, Schneider *et al.*, 2004, Hansen *et al.*, 2007, Bonaldo *et al.*, 2008). This demonstrates that all of the plant protein sources tested could be used successfully to substitute up to 25% of fish meal by weight, with no detrimental effects on growth.

The only feed in either experiment to demonstrate any statistically significant difference in fish performance was the lupin meal feed as offered to cod, which yielded a significant increase in growth rate. Previous studies have also recorded growth enhancement for lupin meal incorporated at up to 50% in feeds for several commercially farmed marine and freshwater fish species (Robaina *et al.*, 1995, Burel *et al.*, 2000a, Pereira and Oliva-Teles, 2004, Glencross *et al.*, 2008). Glencross *et al.* (2002) attributed higher growth among *O. mykiss* receiving a lupin meal-based feed to a poor performance of the fish meal, as opposed to a positive effect of the lupin meal feed. Pereira and Oliva-Teles (2004) attributed enhanced growth performance of *S. aurata* fed micronised lupin meal seed meal to the micronisation and aqueous extraction processes reducing or eliminating antinutrional factors. However, in the current experiments, the lupin meal was not micronised, but was present as whole ground lupin seeds, therefore antinutrional factors would not have been removed by pre-processing. Glencross *et al.* (2002) and Pereira and Oliva-Teles (2004) also noted that lupin meal-fed groups of fish exhibited superior growth performance despite the diets containing reduced levels of methionine (a perceived nutritional limitation of lupin meal). Methionine was also reduced in the current Phase 1 lupin meal feed compared to that in the fish meal feed (data not shown); however these differences did not correspond to differences in cod growth rate.

A trend for an increase in feed intake and FCR with increasing levels of plant protein was noted by Hansen *et al.* (2007) in Atlantic cod, irrespective of protein source. A similar effect was seen in the current Phase 1 experiments, particularly for cod, where the addition of plant proteins resulted in increased feed intake and FCR over the fish meal feed in most cases. This could be due to expected higher levels of indigestible fibre in feeds containing unrefined plant proteins, or to plant protein-derived antinutrional factors damaging the intestinal wall, thereby reducing the efficiency of absorption and leading to an increased need for feed intake to supply the nutritional requirements of maintenance and growth, and the resultant increase in FCR.

If the observed increases in feed intake and FCR were attributable to increased indigestible fibre content, wheat gluten and soy conc. may have been expected to be similar to fish meal, since they are both plant protein extracts rather than meals and

do not have elevated fibre levels; but this was not the case. Regarding possible antinutrional factor-based damage to the GI tract, no enteritis was observed in either species, indeed, evidence of enteritis is rare in studies involving plant protein substitution in feeds for cod and sea bass (Hansen *et al.*, 2006, Refstie *et al.*, 2006, Olsen *et al.*, 2007, Bonaldo *et al.*, 2008). A similar lack of severe enteritis-like changes has also been noted in other species of marine fish, including Egyptian sole (*Solea aegyptiaca*), cobia (*Rachycentron canadum*) and Atlantic halibut (*Hippoglossus hippoglossus*) (Grisdale-Helland *et al.*, 2002, Bonaldo *et al.*, 2006, Romarheim *et al.*, 2008b).

It is also worth noting that the feed intake and FCR values for each fish species were more similar between soy conc. and HiPro soya or LoPro soya than between soy conc. / HiPro soya or LoPro soya and fish meal. The similarity in these parameters between a soy product (specifically soybean meal) and soy conc. was also noted by Schneider *at al.* (2004) in the freshwater species *O. niloticus* at 15% inclusion. Were antinutrional factors playing a significant role in the current study, feed intake and FCR would be expected to be greater for soybean meal (raw meal) compared to soy conc. (processed protein extract, inactivated antinutrional factors). Since this increase in feed intake and FCR was not seen, it is likely that a combination of low level effects led to the observed increase in feed intake and FCR with plant protein inclusion, rather than one major or acute effect.

There were some observed differences in GI tract histo-pathology among feed groups, although these did not equate to plant protein-derived enteritis as reported for other fish species. The reduced muscle thickness observed in the mid intestine of lupin meal-fed cod may be related to the physical instability of the lupin meal feed, which was observed to break down most rapidly in seawater. It is possible that the requirement for physical processing in the gut would therefore be reduced for the lupin meal feed, resulting in reduced muscle thickness. No differences were observed in mid intestine muscle thickness in sea bass, although lupin meal did result in significantly reduced villus diameter for this species. This and other more detailed morphometric differences in the sea bass GI tract (e.g. mid intestine villus surface area and lamina propria diameter, and distal intestine muscle thickness and villus length) are currently unexplained. Regardless of their origin, and due to the

satisfactory growth and performance results, none of the observed differences are considered to represent a detrimental effect of the tested plant proteins for Atlantic cod and European sea bass.

The trends for feed digestibility were comparable among the two fish species, with plant protein-based feeds displaying better performance than fish meal for some digestibility parameters (*note* - ranked data only, statistical testing not possible due to absence of/low numbers of replicates). This aligns with findings from related published studies, where the digestibility of plant proteins was comparable to or better than those of fish meals, for sea bass and cod (Robaina *et al.*, 1999, Tibbetts *et al.*, 2006) and a range of other freshwater and marine fish species. The observed high dry matter and protein digestibility of wheat gluten and soy conc. for both fish species and organic matter digestibility in cod are also as expected, as they are refined protein extracts. For the other tested plant protein sources, the raised levels of indigestible fibre are thought to account for their slightly lower digestibility values.

Published information on the effects of the tested plant protein sources on fish proximate composition is limited, and was only found for lupin meal, soybean meal and soy conc. Lupin meal was shown to result in no differences in proximate composition in feeds for *S. aurata* (Glencross and Hawkins, 2004) or *P. auratus* (Pereira and Oliva-Teles, 2004) at 30% inclusion. Soybean meal also resulted in no differences in *O. niloticus* up to 15% inclusion (Schneider *et al.*, 2004), *G. morhua* up to 16% (Hansen *et al.*, 2007), or *P. olivaceus* and *O. mykiss* up to 40% inclusion (Kikuchi, 1998, Choi *et al.*, 2004, D'Souza *et al.*, 2006). In agreement with these findings, no significant differences in any of these parameters were observed in the current research, i.e. the tested plant proteins did not result in any major changes in body composition up to 25% inclusion in *G. morhua* of *D. labrax*.

The subopercular masses observed at the end of the Phase 1 cod experiment are thought to have been thyroid hyperplasic tissue (Fournie *et al.*, 2005). It is known that reduced intake of iodine can cause such pathology, and it has been suggested that ozonation of seawater may alter the relative concentrations of iodine species in a closed tank system, reducing its bioavailability (Sherrill *et al.*, 2004). However, no subsequent stocks of fish in the same recirculating aquaculture system (RAS), which

incorporates water ozonation, have shown any comparable pathology. The "stargazer" pathology also observed is known to be a problem among intensively reared cod, among which it the most common deformity. It is characterised by an upward bending of the post-cranial vertebrae, resulting in an upward tilt of the neuro-cranium. Studies have shown that on average 47% of intensively reared cod showed this deformity, compared to just 2% in extensively reared cod and absent in wild animals (Fjelldal *et al.*, 2009). Sub-optimal larval nutrition is one possible reason for these differences (Hamre, 2006), and has indeed been shown to have a significant effect on skeletal deformities (Imsland *et al.*, 2006). It is also the case that different genetic stocks of cod can be more or less prone to developing spinal deformities (Kolstad *et al.*, 2006) and it may be that the animals purchased in this case were of a susceptible genetic stock. It is considered likely that the deformities noted were congenital in origin, or at least were caused during early development, and were not as a result of the experimental conditions. Despite the presence of these abnormalities, the growth performance of the cod in the Phase 1 experiment was comparable to published studies and subsequent personal research (Chapter 4), therefore the findings on effects of feeds are deemed to be reliable.

In terms of faecal physical characterisation, particle measurements with the Malvern Mastersizer© do appear to be reproducible (see Chapter 2, Section 2.12.1), although systematic errors owing to an assumption of spherical particles are acknowledged. In taking this assumption into account, the focus therefore is on using the data to obtain a representative and comparative overview of the particle size distributions among feeds and between species within the experiment, as they were all treated and analysed in the same way.

The faecal particle size distributions observed for both fish species contained peaks with similar size ranges to those recorded for fish faecal wastes by previous authors, at around 30, 150, 225 and 540 μm (Maillard *et al.*, 2005, Brinker *et al.*, 2005a, Pfeiffer *et al.*, 2008). The only obvious exception to this was sea bass which did not exhibit a peak at the smallest particle size. This commonality in particle size distribution among different fish species and feed types indicates that there are common elements in the feeds independent of the different test ingredients. In order

to investigate this fully, photo-microscopic examination of the faeces would be required.

There was a discernible horizontal shift in the smallest particle size range to a greater particle diameter for most of the plant protein substituted feeds compared to fish meal, apart from wheat gluten and to a lesser degree lupin meal. A vertical shift is also noticeable in the coarser particle size ranges, where the abundance peaks are higher. Both these shifts suggest that the plant proteins apart from wheat gluten and to a lesser extent lupin meal result in coarser particles than fish meal. The horizontal shift is essentially a more extreme version of the vertical shift, the latter denoting a change in the actual particle size within a peak, the former denoting a change in the abundance of one particle size peak compared to another. A vertical shift is also noticeable in the sea bass particle size distribution, with lupin meal, wheat gluten and fish meal having larger first peaks and smaller subsequent peaks, suggesting a higher abundance of finer particles in these faeces samples. The reasons for these shifts are likely therefore to be due to the protein sources in the feeds, as the differences are common between species; the exact mechanisms are unclear at the present time.

All faecal particle size distributions for both fish species were very platykurtic or non-peaked (Fernandes and Tett, 2001), showing that no one part of the particle size range dominated for either species or for any tested feed. In terms of the skewness of faecal particle size distributions, cod faeces ranged from symmetrical to skewed towards fine particles, while sea bass demonstrated plots ranging from skewed to very strongly skewed towards fine particles (Fernandes and Tett, 2001). These differences between fish species can be readily seen on the particle size distribution plots, as the peaks depicting the coarser particle sizes are larger relative to the peaks depicting the finer particles for cod than sea bass. This is despite the extra peak observed at the finest end of the particle size range seen in the cod particle size distributions. However, the particle size shifts observed on the cod particle size distribution plot do not agree with assumptions drawn from the corresponding skewness data, whereas these data appear to agree with one another in the case sea bass. This discrepancy may be due to the high variability seen in the cod skewness dataset. It should be noted that the particle size distribution analysis of the cod samples was the first particle size measurement run carried out with experimental

samples. As such there may have been a certain amount of operator error due to lack of experience, which was not present by the time the sea bass samples were analysed.

Reviewing the particle size distribution plots from both species and the skewness data from the sea bass, it can be seen that wheat gluten and lupin meal result in finer particles than most other plant proteins, being similar to fish meal. In sea bass, the feed containing whole faba beans resulted in fewer fine faecal particles than that containing fish meal. However, in isolation this information does not provide the whole picture: it is the actual volume or mass of particulate material produced, coupled with this particle size information, which is more applicable. For example, if two feeds both result in very high proportions of fine particulates, but one results in a much lower production of faeces as a whole, then this feed may result in fewer impacts on water treatment efficiencies or on the receiving environment.

The greater abundance of fine particles relative to coarser particles and the higher values of skewness in sea bass faeces compared to the cod faeces suggest that there is more physical and chemical processing of the feeds in the gut of sea bass. It is notable that protein apparent digestibility coefficient was also higher in sea bass than in cod, which supports the suggestion of an increased level of GI tract processing and/or a longer residence time in the gut of sea bass. Previous studies have demonstrated gastrointestinal evacuation time for between 90 and 95% of the GI contents in the range 31 to 46 hr depending on protein source and presence or absence of dietary fillers for *D. labrax* (Langar, 1994, Adin *et al.*, 1998, Adamidou *et al.*, 2009). Detailed information is sparse in the literature for the gastrointestinal evacuation time in *G. morhua*, but from the studies found, it appears to be considerably longer than for sea bass. Dossantos and Jobling (1991) show that around 73 to 89% of gastric content is evacuated in cod after 90 hr, while Hansen *et al.* (2006) found that it took more than 72 hr for a meal to pass through the GI tract. It is therefore suggested that it is not differences in gastrointestinal evacuation time that are affecting particle size distributions or feed digestibility, but some other factor, possibly the intensity of physical processing in the GI tract or its chemical processing that is creating the differences seen. This area warrants further investigation.

The positive relationship between particle size distribution skewness and kurtosis for sea bass faeces means that should a particle size distribution demonstrate a high abundance of fine particles (high/positive skewness) then it will also be less platykurtic (higher kurtosis; relatively more peaked) than a particle size distribution demonstrating a greater abundance of coarser particles (low/negative skewness). This suggests that if a particle size distribution shows a high abundance of fine particles, then these particles will tend to be grouped together in narrow ranges, leading to more pronounced peaks (Fig. 28 (a)). Conversely, if a particle size distribution shows a higher abundance of coarser particles, then these larger particles will be spread across a wider particle size range, flattening the particle size distribution (Fig. 28 (b)).

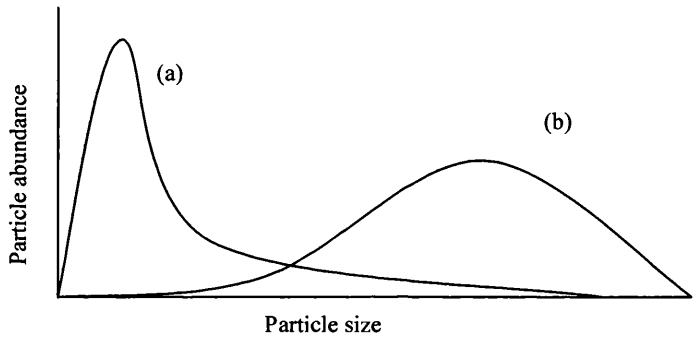

Figure 28: Illustrative representations of a particle size distribution plot showing (a) high skewness (fine particles) and high kurtosis (peaked) in relation to a plot showing (b) low skewness (coarse particles) and low kurtosis (flatter).

In terms of effluent management, this means that faeces demonstrating particle size distributions in which fine particles dominate will result in different treatment challenges to particle size distributions in which coarser particles dominate. In a rearing system incorporating filters, the former situation will firstly result in more faecal particles passing through the filtration stage. Secondly, due to the fines being in a narrow range of particle sizes, if this particle size range is smaller than the filter pore size, then an even greater volume of particles will bypass the filter. In the case of a particle size distribution containing coarser particles, firstly removal efficiency will be higher, and secondly if the pore size is greater than a specific particle size, then a lower proportion of the fine particles will pass through in comparison to the former example, due to the peaks being wider and lower and containing a greater

range of particle sizes. Therefore, coarser particle size distributions in the faeces of sea bass could impart even greater removal efficiency on the effluent than coarser particle size distributions in cod faeces.

For both fish species, plant proteins mostly resulted in greater faecal production than fish meal (statistically significantly so for cod). The notable exception to this was wheat gluten in feeds for sea bass, which produced a lower total quantity of faeces than all other tested feeds. In terms of faecal production, differences were observed between fish species such that cod produced less faeces g kg growth^{-1} than sea bass, irrespective of protein source. Also, in general those feeds in which fish meal had been substituted by plant proteins resulted in greater overall volumes of faeces g kg growth^{-1} in both species. As mentioned previously, the potential errors in the determination of FCR for lupin meal fed cod has resulted in the corresponding cod lupin meal faecal production being omitted. In terms of fine particulate production g kg growth^{-1}, all protein sources resulted in statistically similar fine particulate production in the case of cod. For sea bass, the trend was for least faecal production in all particle size ranges for wheat gluten-fed fish, while lupin meal resulted in the greatest faecal production. The data obtained indicate that the desired reduction in total faecal production and minimization of the volume of fine faecal particles would be accomplished most successfully by substituting fish meal with wheat gluten, particularly in feeds for European sea bass.

The ζ analysis of fine faecal particles from sea bass demonstrated the curve expected of a surface dominated by amino acids as discussed in Chapter 1, Section 1.2.1.2. The data suggests that there will be electrostatic repulsion under farm conditions (i.e. pH in the range 7.5 to 8.0), due to the moderately negative values obtained. However, the faecal suspension would remain within the 'unstable' zone of ζ (where flocculation can occur), between $\pm$ 30 mV. This inherent instability would tend to promote a level of flocculation of the fine particles, leading to a reduction in the fine particle loading with time (Li *et al.*, 2008a). It was supposed that ζ could have an effect on the particle size distribution for this reason, and as such the regression of ζ with particle size distribution kurtosis and skewness was tested. No relationship was observed between ζ and these two parameters. That is not to conclude that ζ does not affect faecal particle size distribution, but rather that the tests carried out herein were

not sufficient to fully investigate this potential relationship. Further tests could be designed to elucidate this, for example by chemically altering the ζ in the laboratory by adjusting pH and testing the particle size distributions at these conditions. Indeed, it may be that only extreme changes in ζ for example outside the ± 30 mV range, will affect the particle size distribution parameters.

As detailed in Chapter 1, published settling velocities for fish faecal particles vary widely and the current data align towards the top end of the published range, at 4.0 and 4.3 cm s^{-1} for *G. morhua* and *D. labrax* respectively. For both fish species, it was calculated that substitution of fish meal with wheat gluten resulted in particles which would tend to remain in the water column for the longest period of time. This means that faecal particles and/or flocs derived from wheat gluten would be more prone to physical breakup or further flocculation (depending on the intensity of the fluid processes). Conversely, substitution of fish meal with LoPro soya and soy conc. resulted in particles that would settle most rapidly out of the tested sources. This means that the faecal particles and/or flocs would have a lower likelihood of being affected by prevailing hydrodynamic conditions, as the particles would not spend as much time in the water column. A general difference was noted in the faba bean feeds fed to sea bass, in that the whole beans resulted in more rapidly settling faeces than the dehulled beans. The rate of faecal settling and its potential impact on particle size changes with time would lead to different management issues depending on aquaculture production system, as discussed below.

The effects of plant protein source on the impacts of effluent management for both tank/raceway based and cage-based fish farming operations can be discussed in the light of the findings from Phase 1 experiments. In RAS and flow through farms, the reduction of fine faecal particulates in feeds containing wheat gluten (associated with low total faecal production , despite a higher proportion of fines), would lessen the quantity of fine particulates bypassing the filtration system and either entering the biofilter (RAS) or being released to the environment (flow through system). However, if settling vessels were a component of the water treatment system, the wheat gluten feed may reduce the amount of settleable faeces due to the high proportion of fine particles. In such a situation, it may be preferable to use a feed that produces intermediate quantities of rapidly settling faeces, such as soy conc. in the

current study. Such a requirement for high density faecal pellets was discussed by Hardy (2000) and Piedrahita (2003) in relation to enclosed or semi enclosed culture systems.

The effects of dietary plant protein source on properties of fish effluents at cage sites are more complex to elucidate. The main parameter of interest is faecal settling characteristics, which are strongly influenced by prevailing hydrodynamic conditions. In general, plant proteins that yield low faecal settling rates (such as wheat gluten in the current study) may be expected to have less impact in the immediate vicinity of cages than those producing coarse particles (e.g. Soy conc.), owing to wide dispersal of fine particles. The desired faecal properties would furthermore be expected to differ for different cage locations, e.g. enclosed low energy sites (lakes, lochs, fjords) versus high energy sites (offshore). Moreover, detailed prediction of settling characteristics in open water systems would require sophisticated models such as those introduced in Chapter 1. However, knowledge of the charge characteristics of fish faeces could allow better predictions of particle and floc interactions during settling (Hargrave, 2003) and impacts on sedimentary characteristics (Milligan and Loring, 1997).

The feed-independent differences in faecal properties among fish species are also worth discussing in relation to different farming situations. Sea bass produced a greater proportion of fine particles than cod irrespective of protein source, and also produce a greater volume of faeces per unit of fish produced, due to the poorer FCR seen in sea bass. Therefore the same implications as highlighted above will be apparent, notably that sea bass would result in a greater amount of waste bypassing filters in comparison to cod, but with a higher mean settling velocity would take less time for a significant proportion of the particle size distribution to settle out of suspension. This would tend to suggest that, when considering faecal properties alone, sea bass would be more suited to land based operations incorporating settlement vessels, or to open water operations where rapid settling faecal matter is required, and cod to land based situations utilising filters, or to open water farms where slow faecal settling is desired.

3.5 Conclusions and considerations for future research

In conclusion, the Phase 1 experiments have shown that any of the plant proteins tested can be safely included at 25% substitution of fish meal by weight for either *Gadus morhua* or *Dicentrarchus labrax,* without significant changes in growth performance. In the former species, using lupin meal as a protein source may in fact increase the growth potential of the fish over fish meal alone. However, considering the wider implications of plant protein substitution, a feed containing 25% lupin meal would result in a greater production of effluent as a whole and as fine particulates per unit of fish growth for both cod and sea bass. For this reason, it is suggested that any of the other plant protein sources would be more suited as fish meal replacers when taking water quality management/ environmental impacts into account. Overall, wheat gluten would be the most suitable candidate as a fish meal replacer, due its high digestibility and reduced faecal output per unit of fish growth, resulting in reduced output in the fine particle size ranges. The wheat gluten feed did however result in a particle size distribution plot more skewed towards fine particles, but this is not considered to be a problem, particularly in land-based RAS or flow through systems, once the total production of fines is taken into account. It may however be an issue in some cage farming situations, where a rapidly settling waste may be more desirable than reduced waste output. Further validation of these results is recommended.

The observed instability imparted on a suspension of fine faecal particles in terms of ζ could potentially be enhanced through the addition of a positively charged material to the feeds. Such a material, if egested with its charge largely unchanged along with the faeces, would act to attract the negatively charged particle/counter ion systems resulting in an increase in flocculation and particle size. This would enhance the filtration efficiency in terms of closed or partially closed culture systems, and further improve the settling properties of the faecal solids as it may allow the proportion of fine particles to be reduced for any feed.

The observed deformities in the Phase 1 cod experiment were of some concern, but having subsequently completed the sea bass experiment with no such effects being recorded, these deformities were assumed to be batch-specific. Indeed, cod have

been shown to be generally susceptible to certain skeletal abnormalities as a result of intensive rearing and poor quality of first feeds. Therefore, no adjustments to the rearing system or water additives were deemed to be necessary for future experiments.

The decision to use auto-feeders (rather than hand feeding to satiation) in the Phase 1 cod experiment was based on lack of a vigorous feeding response and associated difficulty in assessing satiation by direct observation. The limitation of this approach is recognised in terms of reduced accuracy of feed intake measurement. It was anticipated that a higher quality batch of cod in future experiments would resolve this problem, allowing successful hand feeding to satiation. It was also desirable to increase the statistical power and range of analyses in future studies (for both fish species) by increasing the quantities of faecal samples collected.

Specific questions raised by the current research that are desirable to investigate in subsequent experiments are:

- What are the common elements of fish faecal material which appear to be present, independent of plant protein source?

- Does the addition of plant protein affect the stability or rheological properties of the resultant faecal matter?

- Can a material be added to the feeds to achieve the suspension instability required, and will these have any effect on growth or digestibility, and what are their effects on the physical properties of the faecal material?

- What is the makeup of the gut flora in the experimental fish, what is its contribution to faecal mass, and how does it impact upon the particle size distribution of the faecal material? Does protein source influence this flora and can it be manipulated?

Chapter 4

Design and evaluation of feeds for Atlantic cod (*Gadus morhua*) and European sea bass (*Dicentrarchus labrax*) with fish meal substituted by plant proteins with the addition of a potential flocculant (Phase 2)

4.1 Introduction and objectives

Following on from the completion of the feed experiments reported in Chapter 3, it was agreed that due to the overall success of the protein sources from these experiments, the two most commercially viable alternative protein sources would be selected and the inclusion level increased where possible. These were deemed to be HiPro soya and wheat gluten, the decision based on commercial availability and cost. In addition to this, blood meal was discussed as a feed additive that could potentially enhance the physical stability of the faeces (not as a protein source) by acting to neutralise the surface negative surface charge of particles allowing flocculation by Van der Waals forces to occur.

The potential of blood meal as a stability enhancer might be expected as iron in haemoglobin exists as positively charged ions, the reduced soluble divalent ferrous iron (Fe^{+2}) or oxidized insoluble trivalent ferric iron (Fe^{+3}). Due to the positive charge in both states, it may therefore be expected to result in a greater degree of flocculation, based on the zeta potential results found in Chapter 3. This in turn could be expected to alter the particle size distribution, resulting in effects on the effluent removal potential and settlement rate, as it has been shown to do so as an additive to sludges (Adin *et al.*, 1998, Zhou *et al.*, 2008). It was therefore agreed that blood meal would be included at 5% by weight.

Having analysed the particle size distributions and zeta potential of faeces in Phase 1 experiments, an additional analysis was introduced during Phase 2. As discussed in Chapter 2, the stability of faeces can affect the particle size distribution and therefore has implications on processes which are dependent upon this characteristic. For this reason, the rheology of faeces was analysed in order to investigate relationships between protein source and/or blood inclusion and faecal stability. The rheology of faeces has been analysed previously (Brinker *et al.*, 2005b, Brinker, 2007), but in relation to binder effects and not protein source or blood meal addition.

The objectives of the Phase 2 experiments were therefore as follows:

1. Determine whether Atlantic cod and European sea bass can tolerate higher inclusion of HiPro soya in terms of growth and performance;

2. Develop a reliable method for analysing the rheological properties of marine fish faeces based on samples collected by stripping;

3. Investigate whether higher plant protein inclusion and the inclusion of blood meal affects these rheological properties and the previously studies physical properties of fish faeces for European sea bass and Atlantic cod;

4.2 Experimental design

4.2.1 Feeds

A second set of feeds was designed and manufactured according to the method described in Chapter 2, Section 2.4, to supply the nutritional requirements of Atlantic cod and European sea bass. They consisted of fish meal, four feeds with 20% by weight made up of HiPro soya or wheat gluten, both with (20 soy + blood and 20 wheat gluten + blood) and without (20 soy and 20 wheat gluten) 5% blood meal, and two feeds which had 35% HiPro soya with (35 soy + blood) or without (35 soy) 5% blood meal (Table 11).

Table 11: Feed formulations for Phase 2 feeds for cod and sea bass, produced in October 2007. All ingredients reported as g kg^{-1}.

	Fish meal	20 soy	20 soy + blood	35 soy	35 soy + blood	20 wheat gluten	20 wheat gluten + blood
Fish meal	700	577	476	452	406	464	394
Fish oil	79	95	105	95	112	83	105
HiPro soya (48%)	-	200	200	350	350	-	-
Wheat gluten	-	-	-	-	-	200	200
Blood meal	-	-	50	-	50	-	50
Monosodium phosphate	-	-	-	-	-	-	5
L-Lysine	-	-	-	-	-	3	3
DL-Methionine	-	-	-	0.4	1	-	0.3
Vitamin and mineral premix	3	3	3	3	3	3	3
Yttrium oxide premix	1	1	1	1	1	1	1
Wheat starch	218	124	165	100	78	247	240

All feeds were designed to be nutritionally balanced, and as such the higher inclusion of wheat gluten was not possible due to the need to maintain the nutritional balance in the feeds; the proximate analysis of these feeds is shown in Table 12.

Table 12: Proximate composition of Phase 2 feeds for cod and sea bass, g kg^{-1} feed as fed, as per Chapter 2, Section 2.11.

	Fish meal	20 soy	20 soy + blood	35 soy	35 soy + blood	20 wheat gluten	20 wheat gluten + blood
Fat	201	185	180	176	185	171	179
Protein	489	501	495	478	511	505	506
Ash	75	77	71	71	71	58	57
Dry matter	928	907	918	906	922	911	916

4.2.2 Rearing system

As described in Chapter 2, Section 2.1.

4.2.3 Source of animals

Atlantic cod were supplied by Viking Fishfarm Ltd. (Ardtoe, Scotland) and were maintained in quarantine from 8th November 2007 to 4th February 2008. Survival of these animals during transport was 94.51%. Survival rate during quarantine was 88.3%, with most mortality occurring in the first 48 hr after arrival.

European sea bass were those that were used in the previous (Phase 1) experiments. With agreement from the UK Home Office, the animals were reused for the Phase 2 experiment.

4.2.4 Feeding and sampling regime

4.2.4.1 ***Atlantic cod.*** Three days prior to the beginning of the study, 24 juvenile Atlantic cod were randomly assigned to each tank. The source population of cod had been size-sorted in advance and the large and small fish discarded for the purposes of this experiment. The fish were allowed to acclimatise to their new tanks, being fed 3.4 mm Skretting Europa 15© pellets, supplied by Skretting.

On day 0 (13th February, 2008), an initial sample of 21 cod (1 from each tank) was euthanised and measurements made of whole body wet weight (91.44 ± 14.31 g,

mean ± SD), standard length (209.95 ± 8.93 mm, mean ± SD), and excised liver wet weight (7.47 ± 1.87 g; HSI 8.13 ± 1.38 %, mean ± SD) from all fish. Dissected samples of liver and GI tract were fixed in Bouin's solution. The carcasses of all sampled fish were then bagged and frozen for whole body proximate composition analysis. The remaining 23 fish per tank were batch weighed to determine the total biomass in each tank at the start of the experiment.

The experimental fish were fed by hand to apparent satiation for the duration of the growth experiment. Skretting 3.4 mm experiment pellets were fed to the fish up to 90 g individual fish weight following which they were fed the 4 mm experiment pellets. Water temperature was programmed to 13°C (min 12.3°C, max 13.9°C), at a salinity of 30 ppt and a pH of 7.9 to 8.2. Lighting was by halogen lamps programmed to a 12 hr light / 12 hr dark cycle and set to replicate dawn and dusk by rising and lowering gradually over a period of 15 min. Light intensity was approximately 73 lux at the water's surface, averaged across all tanks.

Feed intake was measured and recorded twice weekly, giving average feed consumption data for every day of the week. Tanks were fed from pots containing a known weight of feed, which were weighed twice a week to give feed consumption data for every day of the week. Fish were also batch weighed every fortnight. This data were then used to determine feed intake, SGR and FCR.

Termination of the experiment and collection of final data began on day 97. All fish were caught in tank order and batch weighed to determine endpoint biomass. Six fish per tank were euthanised and their individual weight recorded as well as that of the liver. The fish carcasses, including the remains of the liver, were then bagged and frozen immediately to enable whole body proximate composition analysis. It was deemed at this point unnecessary to take further samples for histological analysis due to the lack of feed related pathology in the Phase 1 experimental fish. Start point samples were also discarded.

In order to expedite the completion of the experiment, those animals graded out and not used in the main experiment were again size sorted to retain the largest animals and entered into a replicated digestibility experiment. This was started on the 28th

March 2008 and consisted of three tanks per feed containing triplicate groups of animals. Due to space constraints two feeds were run in feeding experiments at a time. When one set of two feeds had been completed, fish were switched to a new feed and allowed to acclimatise to the new feed for 1 week prior to being stripped. Production of faeces was found to be very good and as such no special arrangements were required to maximize production. Each tank was equipped with a 12 hr clockwork belt feeder, feeding the fish one meal per day for an hour before artificial sunset. At artificial dawn the following day, all fish were netted from the tank and stripped of their faeces.

Due to the low number of tanks involved, stripping was carried out by a pair of trained members of staff so as to minimise the distress caused to the fish during the procedure. The fish were netted from their holding tanks and anaesthetised to the point of loss of balance. They were then removed from the anaesthetic by hand with the use of a cloth which was held over the head of the fish to reduce stress. Urine was first expelled by placing a gentle pressure slightly anterior to the vent. Fish were then stripped of faeces by placing a light pressure on the abdomen slightly anterior of the vent using forefinger and thumb, and then making movements towards the posterior of the fish. The faeces were dispensed into a 60 ml Sterilin pot and pooled per tank. The fish were then returned to their holding tank where their recovery was observed. This was repeated once per week until sufficient quantity of faeces was collected from each replicate.

Faeces from between 2 and 3 stripping events (depending on the amount produced) were pooled and kept frozen at -30°C pending sub sampling for analysis of particle size distribution, zeta potential, rheological parameters, digestibility, dry matter, protein and amino acid content.

4.2.4.2 ***European sea bass.*** Seven days prior to the beginning of the study, 25 European sea bass were randomly assigned to each tank. No samples were taken at the start of this experiment as the aim was only to collect faeces for later analysis. The fish were allowed to acclimatise to their new tanks, being fed 3.4 mm Skretting Europa 15© pellets. The replicated faecal collection experiment was carried out in

November and December 2007 with a start body weight of 449.61 ± 55.48 g, mean ± SD.

The fish were fed Skretting 4 mm experiment pellets to excess using a single 12 hr timed belt feeder per tank for the duration of the experiment. Water temperature was programmed to 24°C, at a salinity of 30 ppt and pH of 7.9 to 8.2. To ensure that the fish were stripped during the peak of faecal production following feeding, i.e. approximately 9.0 to 10.5 hr after feeding (Dias *et al.*, 1998), lighting was programmed to a 15.5 hr light/ 8.5 hr dark cycle, with the fish being fed one meal per day for an hour before sunset. At dawn the following day, all fish were netted from the tank, and stripped of their faeces. Light intensity was approximately 73 lux at the water's surface.

Stripping was carried by a team of trained members of staff so as to minimise the distress caused to the fish during the procedure. The fish were netted from their holding tanks and anaesthetised to the point of loss of balance. They were then removed from the anaesthetic by hand with the use of a cloth which was held over the head of the fish to reduce stress. Urine was first expelled by placing a gentle pressure slightly anterior to the vent. Fish were then stripped of faeces by placing a light pressure on the abdomen slightly anterior of the vent using forefinger and thumb, and then making movements towards the posterior of the fish. The faeces were dispensed into a 60 ml Sterilin pot and pooled per tank. The fish were then returned to their holding tank where their recovery was observed. This was repeated once per week until sufficient quantity of faeces was collected from each replicate.

Faeces from all stripping events over a period of approximately 12 weeks were pooled and kept frozen at -30°C pending sub sampling for analysis of particle size distribution , zeta potential, rheological parameters, digestibility, dry matter, protein and amino acid content.

4.2.5 Sample analyses

All sample analyses, data handling and statistical analyses were carried out in accordance with the methods detailed in Chapter 2.

4.3 Results

4.3.1 Atlantic cod

4.3.1.1 *Survival, growth, feed utilisation and whole body proximate composition.*

The mean cumulative survival rate of Atlantic cod across all feed groups was 95.7% with no statistically significant differences observed among diets. Final body weight, body weight increase, SGR, FCR and feed intake data are summarised in Table 13. Data for each of these parameters were determined to be normally distributed; no statistically significant differences were found among feed groups for any of these parameters. Mean final body weight ranged from 191.86 to 204.75 g, body weight increase from 0.95 to 1.14 g day^{-1}, SGR from 0.73 to 0.80% b. wt. day^{-1}, FCR from 0.96 to 1.08 and feed intake from 0.72 to 0.84%. Hepatosomatic index (HSI) was also found to be normally distributed, with homogenous variance and no violation of the homogeneity of regression slopes. No statistically significant differences in HSI were observed among feed groups, with an overall mean value of 8.69%.

End point values for whole body proximate composition (as g kg^{-1} live weight) are summarised in Table 14. All proximate composition data were normally distributed, with statistically significant differences being found among feed groups for ash content. Atlantic cod receiving the 20 wheat gluten + blood feed contained significantly less ash (25 g kg^{-1}) than those receiving 20 soy, 35 soy, 35 soy + blood or 20 wheat gluten (at 30, 32, 31, and 31 g kg^{-1} respectively).

Table 13: Fish growth, feed utilisation and hepatosomatic index data for Atlantic cod fed Phase 2 experimental feeds for 97 days. Values are mean ± standard deviation. No statistically significant differences among diets were observed for any of the parameters ($p > 0.05$). † Determined from total tank biomass (= "batch weight") for each of three replicate tanks; ▲ $n = 18$ from individual weighing (6 fish per tank).

	Fish meal	20 soy	20 soy + blood	35 soy	35 soy + blood	20 wheat gluten	20 wheat gluten + blood
Initial body weight (g)†	94.75 ± 1.38	96.96 ± 3.50	91.23 ± 1.70	92.25 ± 2.78	93.52 ± 1.87	91.10 ± 2.16	91.83 ± 3.89
Final body weight (g)†	203.81 ± 47.87	192.83 ± 51.31	199.87 ± 51.45	195.76 ± 50.86	191.86 ± 49.61	204.75 ± 44.24	201.91 ± 47.09
Body weight increase (g day^{-1})†	1.04 ± 0.09	1.14 ± 0.24	1.09 ± 0.10	0.95 ± 0.07	1.07 ± 0.14	1.08 ± 0.06	1.05 ± 0.16
Specific growth rate (% b.wt. day^{-1})†	0.73 ± 0.09	0.80 ± 0.08	0.75 ± 0.03	0.73 ± 0.03	0.77 ± 0.08	0.74 ± 0.06	0.76 ± 0.06
Feed conversion ratio†	0.96 ± 0.09	1.03 ± 0.06	1.08 ± 0.03	1.08 ± 0.09	1.03 ± 0.07	1.02 ± 0.05	1.03 ± 0.07
Feed intake (% b.wt. day^{-1})†	0.72 ± 0.06	0.84 ± 0.04	0.83 ± 0.01	0.81 ± 0.06	0.80 ± 0.06	0.77 ± 0.03	0.80 ± 0.02
Hepatosomatic index (HSI)▲	9.16 ± 1.67	8.22 ± 2.21	8.74 ± 2.14	7.99 ± 2.26	8.21 ± 1.89	8.88 ± 1.54	9.64 ± 1.77

Table 14: Proximate composition (g kg^{-1} live weight) of whole ground Atlantic cod carcasses after receiving Phase 2 experimental feeds for 97 days. Values are mean ± standard deviation. Different superscript letters signify statistically significant differences among diets ($p < 0.05$).

	Fish meal	20 soy	20 soy + blood	35 soy	35 soy + blood	20 wheat gluten	20 wheat gluten + blood
Protein g kg^{-1}	160 ± 7	164 ± 5	162 ± 13	162 ± 2	158 ± 4	164 ± 3	147 ± 7
Lipid g kg^{-1}	77 ± 8	67 ± 12	74 ± 7	67 ± 5	67 ± 7	70 ± 0	75 ± 5
Ash g kg^{-1}	27 ± 1^{ab}	30 ± 2^{a}	28 ± 3^{ab}	32 ± 4^{a}	31 ± 4^{a}	31 ± 1^{a}	25 ± 1^{b}
Dry matter g kg^{-1}	265 ± 2	261 ± 15	266 ± 14	261 ± 11	256 ± 12	267 ± 6	249 ± 8

Feed digestibility data are summarised in Table 15. Data for protein digestibility were normally distributed and were tested parametrically. Both dry matter and organic matter digestibility data were non-normally distributed and were tested non-parametrically, since no transformation was possible. Statistically significant differences were found among feed groups for each of the tested digestibility parameters. Protein digestibility was significantly enhanced by the addition of wheat gluten over all other feeds, followed by fish meal which in turn resulted in significantly better protein digestibility than feeds containing 35 soy, 20 soy or 35 soy + blood. The latter two feeds showed significantly poorer protein digestibility than all other feeds.

Table 15: Apparent digestibility of Phase 2 experimental feeds (dry matter, organic matter and protein) for Atlantic cod. Values are mean ± standard deviation. Different superscript letters signify statistically significant differences among feed groups ($p < 0.05$).

	Dry matter %	Organic matter %	Protein %
Fish meal	77.2 ± 0.4	80.4 ± 0.4	86.1 ± 0.5^{b}
20 soy	66.9 ± 0.5	70.3 ± 0.5	81.1 ± 0.1^{d}
20 soy + blood	76.6 ± 2.5	78.5 ± 2.3	85.2 ± 1.5^{bc}
35 soy	68.0 ± 2.7	70.5 ± 2.5	84.5 ± 0.5^{d}
35 soy + blood	65.3 ± 0.5	67.8 ± 0.4	82.1 ± 0.3^{d}
20 wheat gluten	78.3 ± 0.9	80.4 ± 0.9	88.7 ± 0.2^{a}
20 wheat gluten + blood	78.1 ± 0.5	79.6 ± 0.5	87.7 ± 0.2^{a}

There was insufficient statistical power to detect individual treatment differences by pairwise comparison for the dry matter and organic matter datasets. Metrics in the means ranks from the Kruskal Wallis (KW) test were therefore used to explore where

the differences might lie. dry matter digestibility mean ranks suggest that both wheat gluten feeds are similar with high dry matter digestibility, followed by fish meal and 20 soy + blood, then 20 soy and 35 soy, with 35 soy + blood having the lowest dry matter digestibility, dissimilar to any of the other feeds. organic matter digestibility mean ranks show a slightly different pattern, with 20 wheat gluten and fish meal feeds being similar with high organic matter digestibility, followed by 20 wheat gluten + blood and 20 soy + blood, then 20 soy and 35 soy, with 35 soy + blood having the lowest organic matter digestibility, dissimilar to any other feeds.

4.3.1.2 *Faeces.*

Particle size distribution

As shown in Figure 29, all Phase 2 feeds resulted in similar shaped faecal particle size distribution profiles for Atlantic cod, with all observed frequencies being homogeneous ($p < 0.01$). Three main peaks were observed, common among all feeds at approximately 92, 230, and 460 µm.

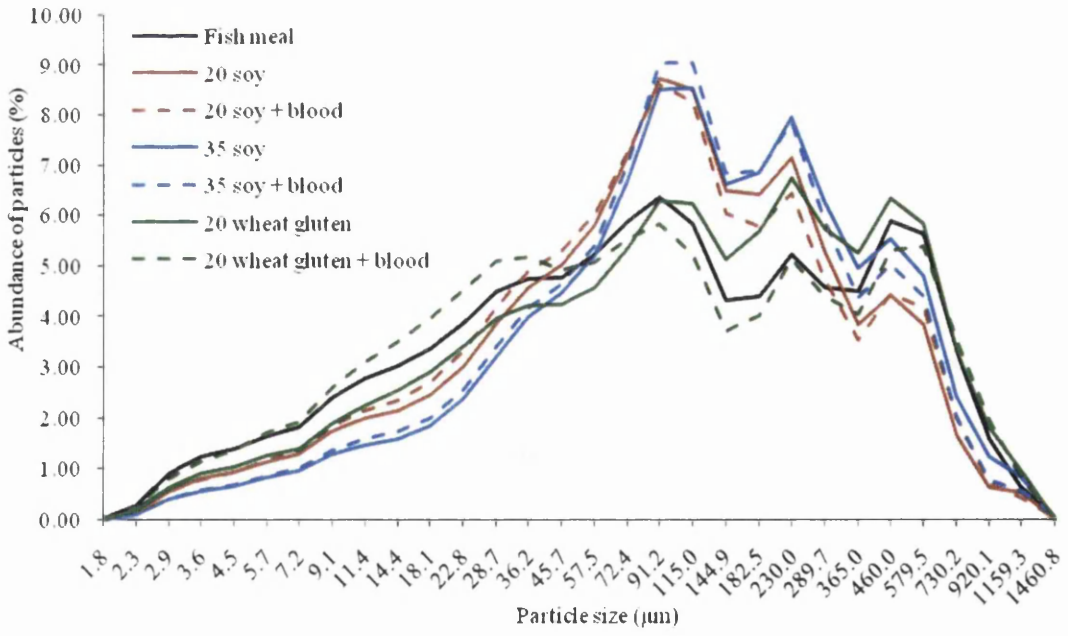

Figure 29: Particle size distributions of faeces produced by Atlantic cod fed the Phase 2 experimental feeds.

Results for particle size distribution kurtosis and skewness are summarised in Figure 30. The kurtosis dataset was found to be normally distributed, and demonstrated no

statistically significant differences among feeds. The skewness dataset was non-normally distributed and, due to containing both positive and negative values, was not transformed but analysed non-parametrically. There was insufficient statistical power to detect individual treatment differences by pairwise comparison for particle size distribution skewness. Metrics in the means ranks from the KW test were therefore used to explore where the differences might lie.

All feeds demonstrated highly platykurtic plots (negative values, relatively flat plots), suggesting little dominance of any one particle size range. Skewness mean ranks for 35 soy + blood, 20 soy, 35 soy, and 20 soy + blood grouped together, as reflected in the skewness values for these feeds (0.57, 0.49, 0.45 and 0.40 respectively). Faeces from cod receiving the wheat gluten, fish meal and WGB feeds displayed negative skewness (-0.03, -0.19 and -0.43 respectively) and this is reflected in their similar mean ranks.

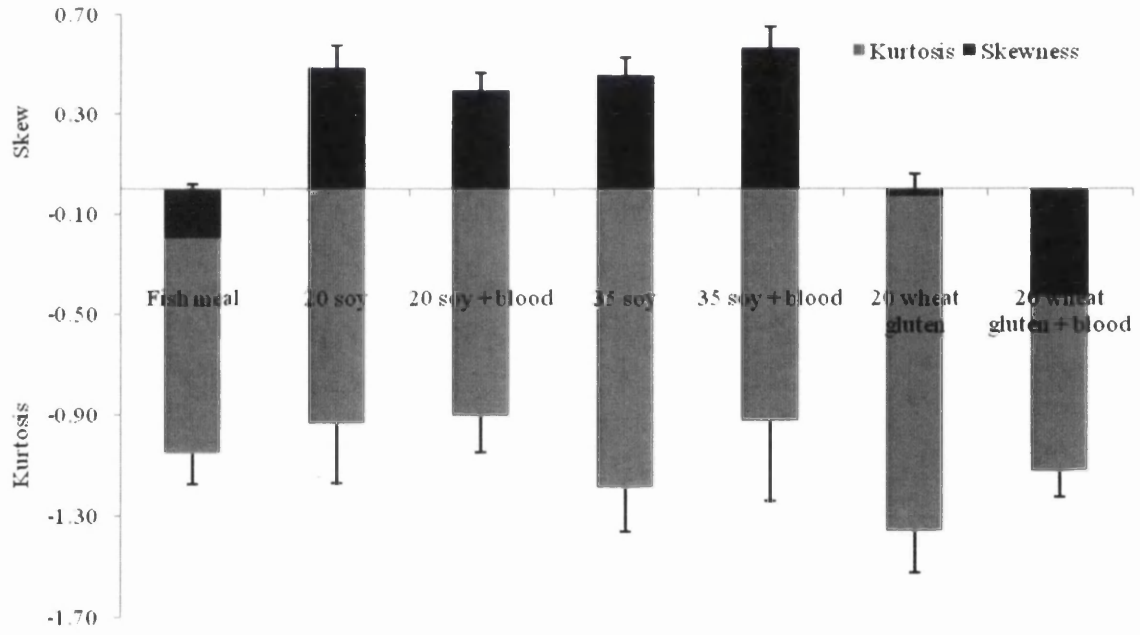

Figure 30: Kurtosis and skewness of faeces particle size distributions derived from the faeces of Atlantic cod fed Phase 2 experimental feeds. Values are mean ± standard deviation. No statistically significant differences were noted ($p > 0.05$).

Regression of kurtosis against skewness (Figure 31) showed that there was no statistically significant relationship between the two parameters for Atlantic cod ($F_{1, 19} = 3.089$, $r^2 = 0.095$, $p > 0.05$).

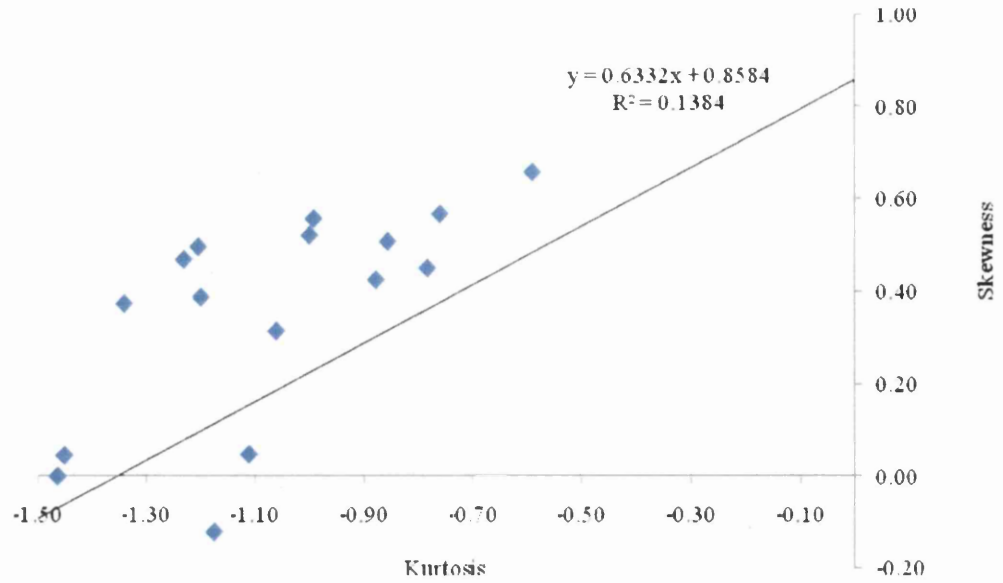

Figure 31: Scatter plot and linear regression of faecal particle size distribution kurtosis against skewness to determine any relationships between the two parameters in the case of Atlantic cod receiving Phase 2 experimental feeds.

Zeta potential of faecal particles

For completeness, the combined result of the three replicates of ζ from each feed and the mean of these results are shown in Figure 32. This shows that the ζ of the faeces is negative for the higher range of pH. IEP is achieved at pH 4.62. The plot shows a relatively rapid decline in ζ between pH 2 and 7, followed by a less rapid decline in the ζ between pH 7 and 9.8, followed by a steepening of the decline once again.

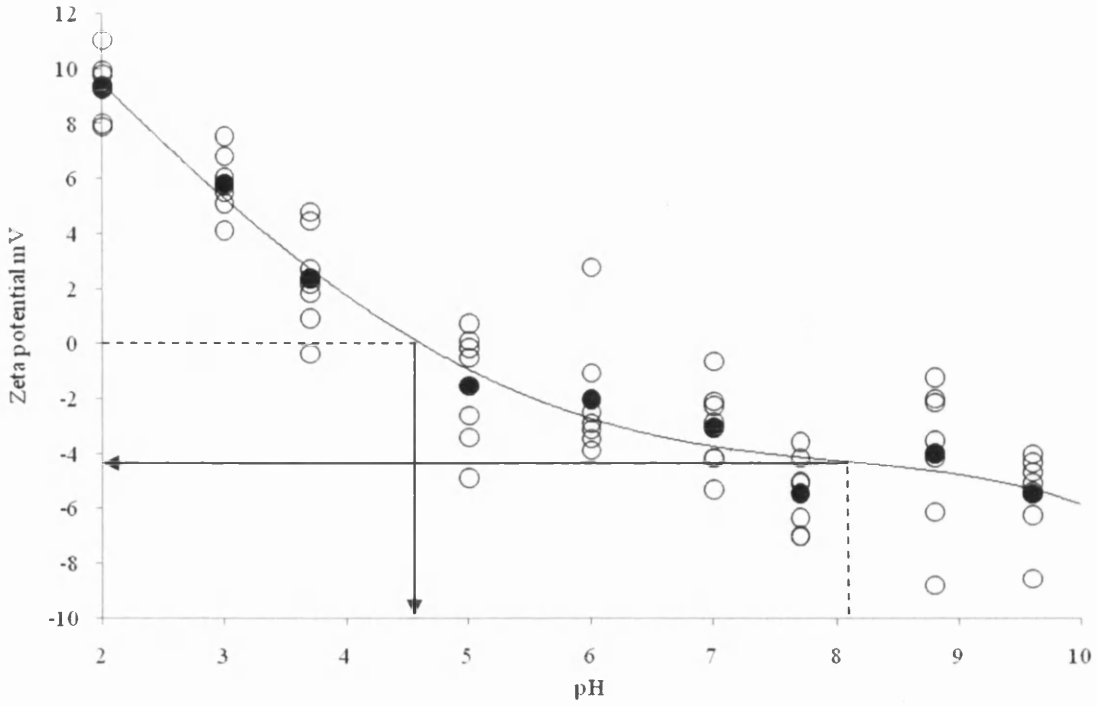

Figure 32: Combined zeta potential (ζ) plot of faeces from Atlantic cod fed Phase 2 experimental feeds. Trend line is fitted to the mean values, denoted by filled circles. The position of the combined isoelectric point (IEP) is shown, and is calculated as pH 4.62. The ζ at pH 8.05 was calculated as -4.07 for the combined data.

ζ and IEP at the system operating pH of 8.05 are shown for each feed in Table 16. Data for both parameters were normally distributed, and statistically significant differences were observed among feed groups for both parameters. ζ at pH 8.05 ranged from a high of -1.60 mV for faeces from fish fed fish meal (significantly different from 20 soy, 35 soy, and 35 soy + blood) to a low of -7.12 mV in the case of 35 soy + blood, which was significantly different from 20 wheat gluten, fish meal and 20 soy + blood. IEP ranged from pH 4.0 for fish meal (significantly different from 20 soy + blood, 35 soy, 35 soy + blood and 20 wheat gluten + blood) to pH 5.5 for 20 wheat gluten + blood (significantly different from 20 soy, 35 soy and 35 soy + blood).

Regression of ζ at pH 8.05 against the particle size distribution kurtosis showed that there was no statistically significant relationship between the parameters ($F_{1, 19}$ = 0.062, r^2 = 0.003, $p > 0.05$). There was however a statistically significant but weak relationship between ζ at pH 8.05 and the particle size distribution skewness ($F_{1, 19}$ = 5.517, r^2 = 0.225, $p < 0.05$).

Table 16: Zeta potential (ζ) in millivolts (mV) at rearing system pH (pH 8.05) and isoelectric point (IEP) for faeces produced by Atlantic cod fed Phase 2 experimental feeds. Values are mean ± standard deviation. Statistically significant differences are denoted by different superscript letters ($p < 0.05$).

	ζ @ pH 8.05	IEP
Fish meal	-1.60 ± 0.70^{c}	4.02 ± 0.14^{d}
20 soy	-4.99 ± 0.57^{ab}	4.46 ± 0.30^{bcd}
20 soy + blood	-3.29 ± 0.69^{bc}	5.71 ± 0.25^{a}
35 soy	-5.57 ± 1.27^{ab}	5.07 ± 0.62^{abc}
35 soy + blood	-7.12 ± 2.83^{a}	5.29 ± 0.61^{ab}
20 wheat gluten	-3.62 ± 2.76^{bc}	4.31 ± 0.50^{cd}
20 wheat gluten + blood	-4.17 ± 0.17^{abc}	5.48 ± 0.76^{a}

Calculated faecal production

Faecal production (Fig. 33) was determined as described in Chapter 2, Section 2.15. Data for faecal production in the "up to 102 μm" and "up to 256 μm" particle size ranges were normally distributed, whereas data for total production of faeces in terms of kg^{-1} growth were non-normally distributed and were tested non-parametrically as no transformation was possible .

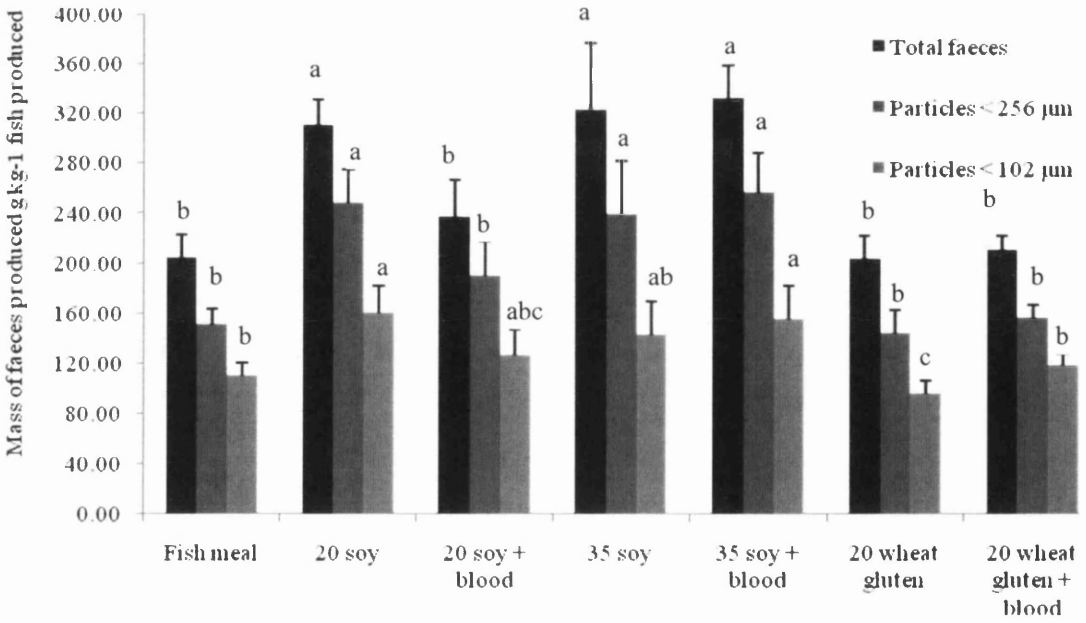

Figure 33: Mass of total faeces, faecal particles below 102 μm and faecal particles below 256 μm produced (g kg^{-1} growth) by Atlantic cod receiving Phase 2 experimental feeds. Values are mean ± standard deviation. Different letters signify statistically significant differences ($p < 0.05$).

The 35 soy + blood, 35 soy and 20 soy feeds produced significantly more total faeces g kg^{-1} growth (at 332, 323 and 311 g respectively) than all other feeds (Fig. 33). The same trend and statistically significant differences among feeds were found for faecal particles up to 256 μm, with the 35 soy + blood, 35 soy and 20 soy feeds producing more particles g kg^{-1} growth than all other feeds (at 256, 239 and 249 g respectively). For particles up to 102 μm, each of the feeds containing soy yielded significantly more particles g kg^{-1} growth than any of the other feeds tested.

Faeces settling velocity

The mean settling velocity of Atlantic cod faecal particles calculated from particle size and density was 3.2 cm s^{-1} (overall mean for all replicates from all Phase 2 feeds, as no statistically significant differences were found among feeds). This value was then used to determine the time taken to settle in 10 cm of water, as detailed in Chapter 2, Section 2.16.

The settlement data are presented in two plots, split to allow better resolution at the lower end of the x axis, the first (Fig. 34) representing 10 to 70% of the distribution, and the second (Fig. 35) being 70 to 95% of the distribution. The data for the 0 to 10% fraction of the particle size distribution is excluded from the dataset due to this being made up of the coarsest particles in the distribution and as such they would be expected to settle out of suspension almost instantaneously. The 95 to 100% fraction of the distribution by contrast would be expected to remain in suspension indefinitely, due to their being the finest particles in the distribution, and as such are also excluded from the dataset. Data for faeces settlement time at both the 50% and 90% fraction of distribution were found to be non-normally distributed, and were tested non-parametrically as no transformation was possible.

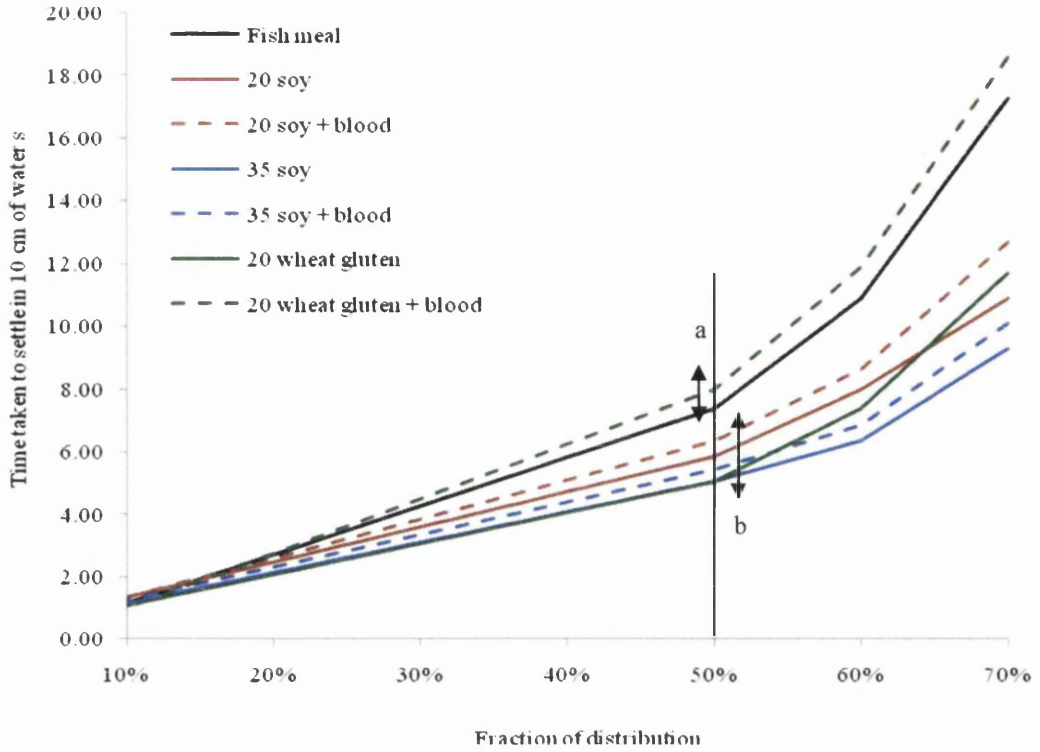

Figure 34: Calculated time taken (s) for Atlantic cod faecal particles in the range 10 to 70% of the total particle size distribution (from coarse to fine particles) to settle in 10 cm of water. Different letters (grouped) signify statistically significant differences ($p < 0.05$).

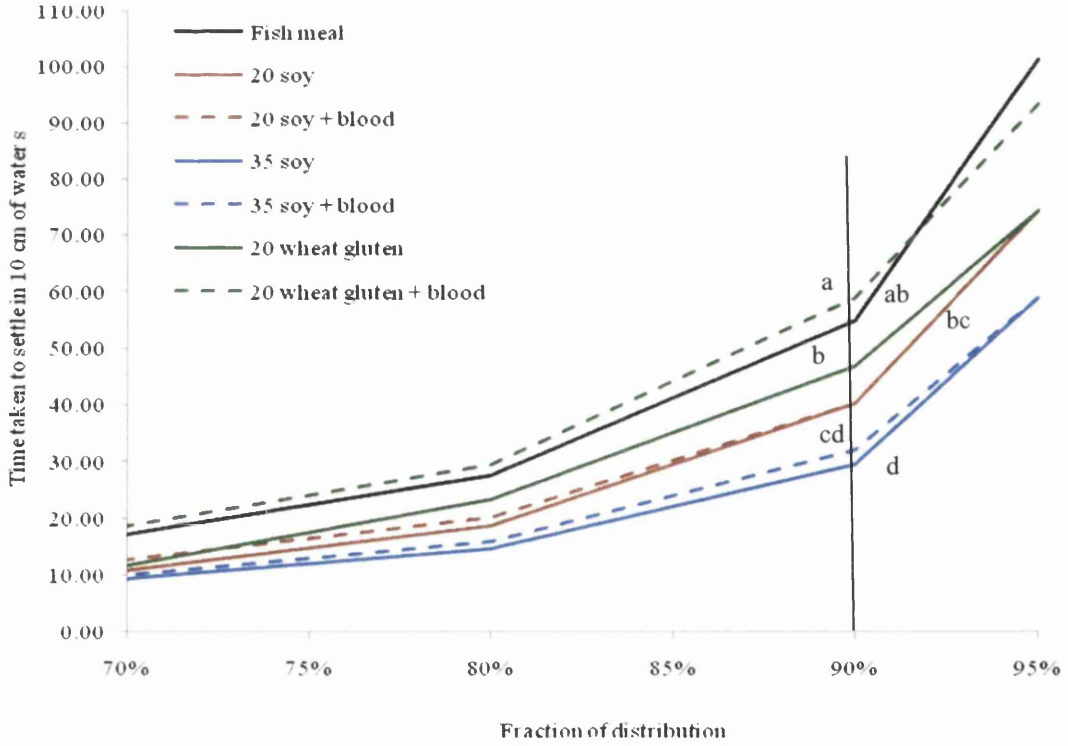

Figure 35: Calculated time taken (s) for Atlantic cod faecal particles in the range 70 to 90% of the total particle size distribution (from coarse to fine particles) to settle in 10 cm of water. Different letters signify statistically significant differences ($p < 0.05$).

Statistically significant differences were observed among feeds at both the 50% and 90% fractions of the distribution. At the 50% fraction of the distribution, the 20 wheat gluten + blood and fish meal feeds yielded faeces with significantly slower settling rates than for all other feeds. Similar trends were observed at the 90% fraction of the distribution, although the differences between the slowest settling (fish meal, 20 wheat gluten + blood) and fastest settling (35 soy, 35 soy + blood) faeces were more extreme.

Faecal rheological characteristics

The rheological data were analysed by plotting the $\log_{10}$ complex viscosity (Pa.S), the main effect, against $\log_{10}$ frequency (Hz) derived from the frequency sweep analysis (Chapter 2, Section 2.17) and were analysed as per Chapter 2, Section 2.18. Variances were found to be unequal ($p < 0.0001$), and statistically significant differences were recorded between feed treatments ($p < 0.0001$) for the main effect (Fig. 36). There was however also a significant interaction term (tank * feed) indicating violation of assumption of homogeneity of regression slopes, suggesting inter-tank/intra-feed differences. Due to this and the unequal variance noted, the model is deemed to be unsound in this case as there are differences within tanks exposed to the same experimental feed.

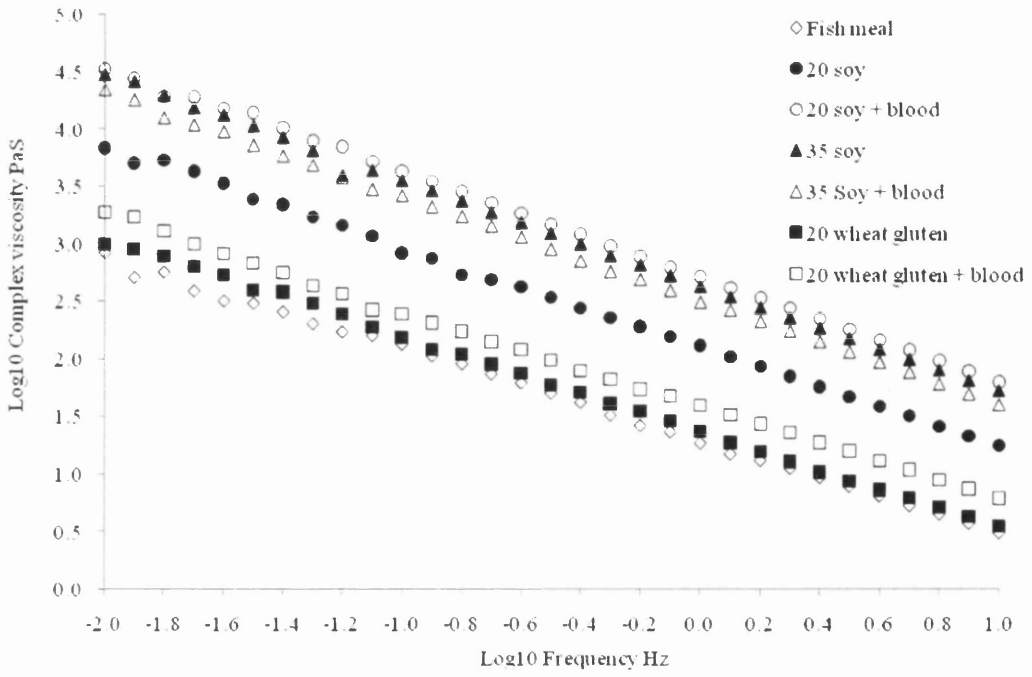

Figure 36: Frequency sweep data from the faeces of Atlantic cod fed the Phase 2 feeds.

Table 17: Mean viscosity (Pa.S ± standard deviation) of faecal material produced by Atlantic cod fed the Phase 2 experimental feeds (n = 3).

	Mean viscosity Pa.S
Fish meal	142.4 ± 16.8
20 soy	1245.5 ± 860.1
20 soy + blood	5817.3 ± 2406.1
35 soy	5070.3 ± 1414.1
35 soy + blood	3552.9 ± 1300.0
20 wheat gluten	200.6 ± 65.1
20 wheat gluten + blood	334.7 ± 94.3

The mean viscosity of faecal material is shown in Table 17. The data were found to be non-normally distributed and untransformable. Non-parametric analysis showed that there were significant differences in the data. However, as will be discussed subsequently, the data are thought to be erroneous (as exemplified by the extremely large values of standard deviation) and as such the differences are not shown. As viscosity is generally agreed to be an analogue of faecal stability, the approximate trend from least to most stable as determined from Figure 36 and Table 17 is as follows:

> Fish meal < 20 wheat gluten < 20 wheat gluten + blood < 20 soy < 35 soy + blood < 35 soy < 20 soy + blood

The fact that statistically significant differences were measured in the dynamic data (Fig. 36) supports the assumptions made from the visual analysis i.e. the stability ranking, although pairwise post hoc analysis is not possible due to the violation of the assumption of homogeneity of regression slopes.

The storage or elastic modulus (G') and loss or viscous modulus (G'') data demonstrates that the inclusion of plant proteins increased both G' and G'' over the values obtained using fish meal (Fig. 37). Similar results were obtained for most plant proteins at an increase of approximately an order of magnitude. This demonstrates that the addition of plant proteins results in faeces which are more viscous and have a greater elastic component, i.e. they are more solid like, and so more resistant to shear forces.

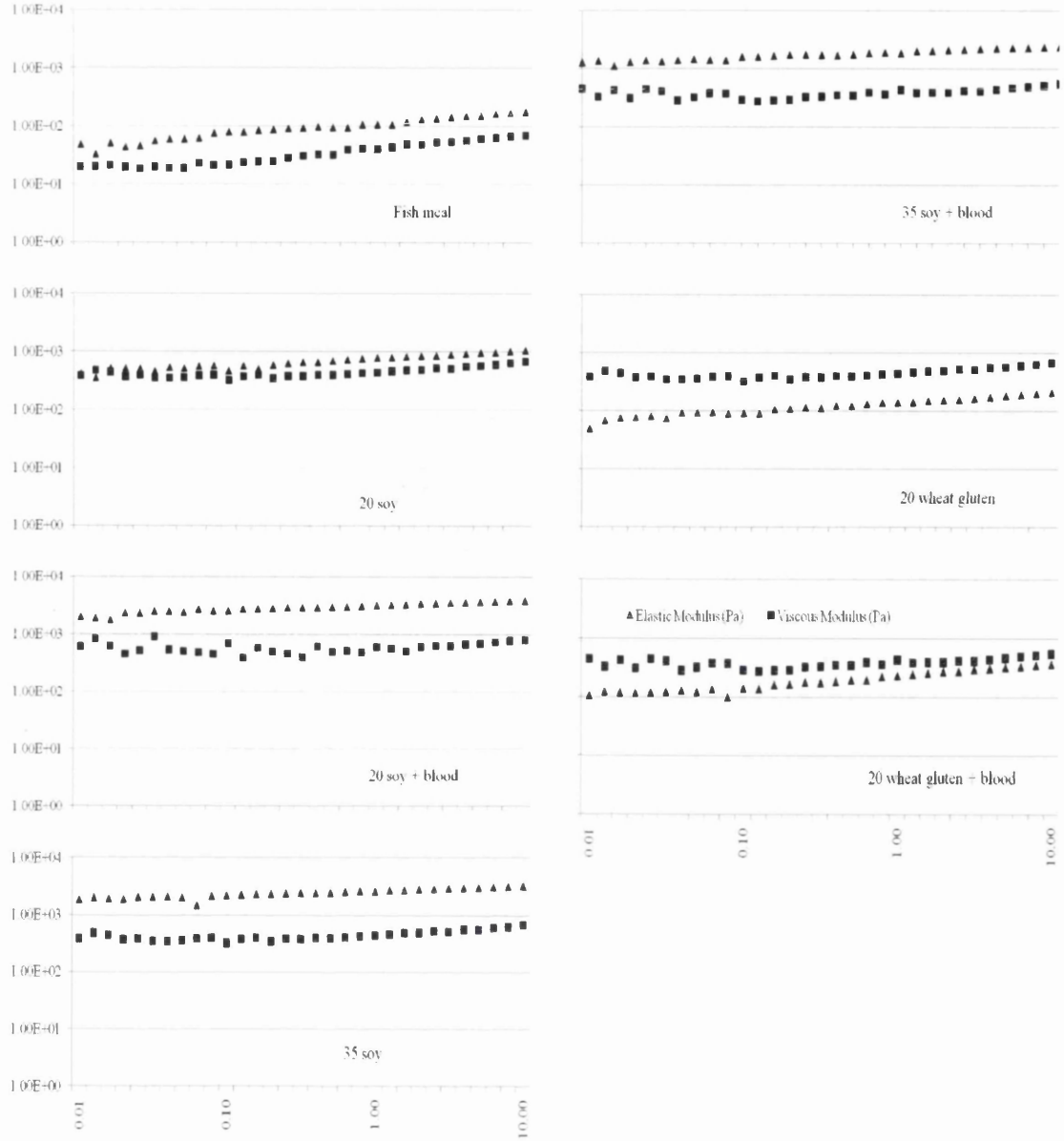

Figure 37: The storage modulus (elastic modulus, G') and loss modulus (viscous modulus, G'') as a function of oscillatory frequency, for the faeces of Atlantic cod fed Phase 2 experimental feeds.

No statistical analysis was identified which could satisfactorily test the data for statistical significance of differences, and thus no analysis was undertaken but the known relationships described. Most plots also show the G'' to be higher in value than the G', showing that these fish faeces irrespective of protein source have a more prominent elastic behaviour than viscous behaviour. However, both wheat gluten feeds resulted in faeces demonstrating the opposite relationship, where G' is higher

than G'', suggesting that these faeces were more 'watery' having a more prominent viscous modulus in comparison to their elastic modulus.

4.3.2 European sea bass

4.3.2.1 ***Feed utilisation.*** No growth data were collected as it was agreed that carrying out growth measurements on sea bass would not add significantly to the current literature, and the determination of faecal characteristics was of more immediate concern.

Mean cumulative survival rate of European sea bass across all feed groups was 99.8% with no statistically significant differences observed among diets. Feed digestibility data are summarised in Table 18. Data for protein digestibility were normally distributed and were tested parametrically. Both dry matter and organic matter data were non-normally distributed and dry matter was tested non-parametrically, since no transformation was possible. Organic matter was successfully transformed and as such was tested parametrically. Statistically significant differences were found among feed groups for the protein digestibility and dry matter digestibility parameters. No statistically significant differences were noted in the organic matter digestibility parameter. Results of pairwise comparisons of protein digestibility are shown in Table 18. protein digestibility was significantly enhanced by the addition of 20 wheat gluten + blood, 20 soy and 20 soy + blood over all other feeds, followed by fish meal. 35 soy and 35 soy + blood which in turn resulted in significantly better protein digestibility than feeds containing 20 wheat gluten. The latter feed showed significantly poorer protein digestibility than all other feeds.

Table 18: Apparent digestibility of Phase 2 experimental feeds (dry matter, organic matter and protein) for European sea bass. Values are mean ± standard deviation. Different superscript letters signify statistically significant differences among feed groups ($p < 0.05$).

	Dry matter %	Organic matter %	Protein %
Fish meal	70.0 ± 3.8	79.4 ± 1.8	88.3 ± 0.8^{b}
20 soy	82.2 ± 3.7	86.9 ± 2.0	93.1 ± 1.4^{a}
20 soy + blood	75.6 ± 2.8	81.5 ± 2.0	91.8 ± 1.1^{a}
35 soy	70.4 ± 1.6	78.7 ± 1.1	88.7 ± 0.6^{b}
35 soy + blood	68.5 ± 1.4	82.9 ± 12.5	89.4 ± 1.3^{b}
20 wheat gluten	67.6 ± 1.6	77.7 ± 1.0	86.1 ± 0.9^{c}
20 wheat gluten + blood	85.7 ± 1.1	90.5 ± 0.7	93.3 ± 0.8^{a}

There was insufficient statistical power to detect individual treatment differences by pairwise comparison for the dry matter dataset. Metrics in the means ranks from the KW test were therefore used to explore where the differences might lie. dry matter digestibility mean ranks suggest that both 20 soy feeds and 20 wheat gluten + blood are similar with high dry matter digestibility, with the remaining feeds resulting in dry matter digestibility that did not differ to any great degree from each other.

4.3.2.2 *Faeces.*

Particle size distribution

As shown in Figure 38, Phase 2 feeds resulted in similar shaped faecal particle size distributions for European sea bass, with all observed frequencies being homogeneous ($p < 0.01$). Three main peaks were observed, common among all feeds at approximately 105, 255, and 645 μm.

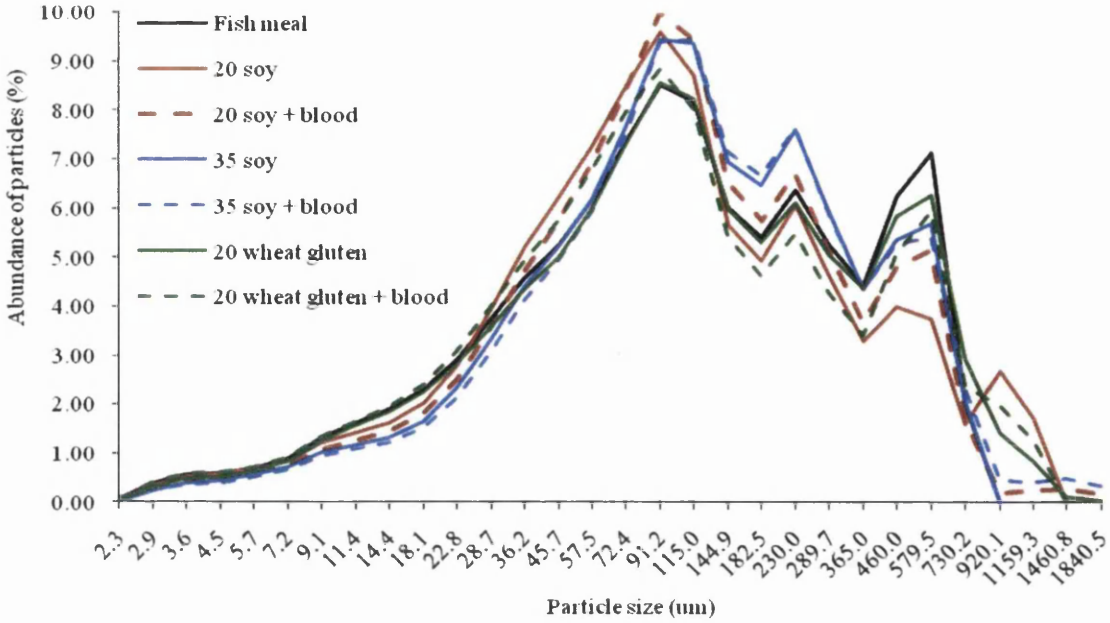

Figure 38: Particle size distributions of faeces produced by European sea bass fed the Phase 2 experimental feeds.

Results for particle size distribution kurtosis and skewness are summarised in Figure 39. Both datasets were found to be normally distributed, and the skewness dataset demonstrated statistically significant differences among feeds. All feeds demonstrated highly platykurtic plots (negative values, relatively flat plots), suggesting little dominance of any one particle size range. Skewness was found to be greatest for the 20 soy + blood feed, significantly greater that for fish meal. This feed also resulted in significantly lower skewness than for both the 20 soy and 35 soy + blood feeds. No other statistically significant differences were noted.

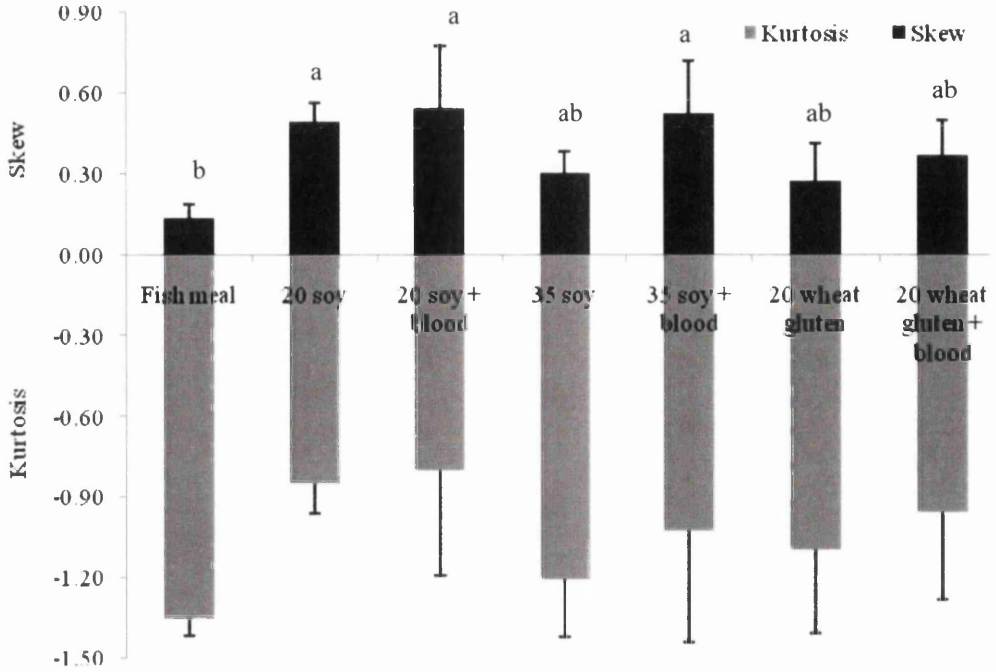

Figure 39: Kurtosis and skewness of faeces particle size distributions derived from the faeces of European sea bass fed Phase 2 experimental feeds. Values are mean ± standard deviation. Statistically significant differences are denoted by different letters ($p < 0.05$).

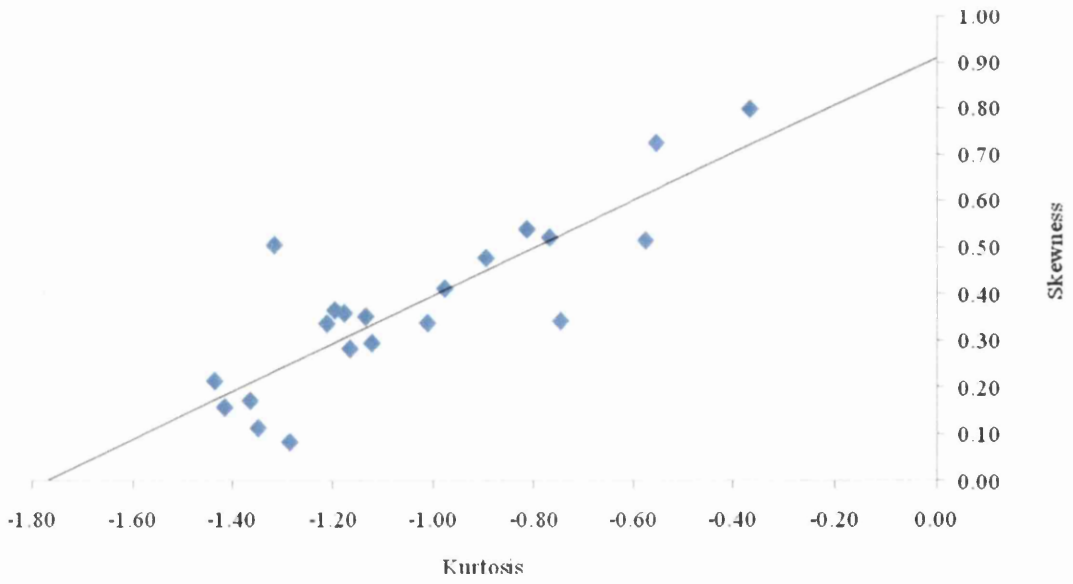

Figure 40: Scatter plot and linear regression of faecal particle size distribution kurtosis against skewness to determine any relationships between the two parameters in the case of European sea bass receiving Phase 2 experimental feeds.

Regression of kurtosis against skewness (Fig. 40) showed that there was a highly statistically significant relationship between the two parameters for European sea

bass ($F_{1, 19} = 47.448$, $r^2 = 0.714$, $p < 0.001$) demonstrating that the coarser the particles (lower value of skewness) the more platykurtic the distribution becomes (more negative value of kurtosis).

Zeta potential of faecal particles

For completeness, the combined result of the three replicates of ζ from each feed and the mean of these results are shown in Figure 41. This shows that the ζ of the faeces is negative for the higher range of pH. IEP is achieved at pH 5.01. The plot shows a relatively rapid decline in ζ between pH 2 and pH 7.5, followed by an increase in the ζ to pH 9.5, and a subsequent drop at higher pH.

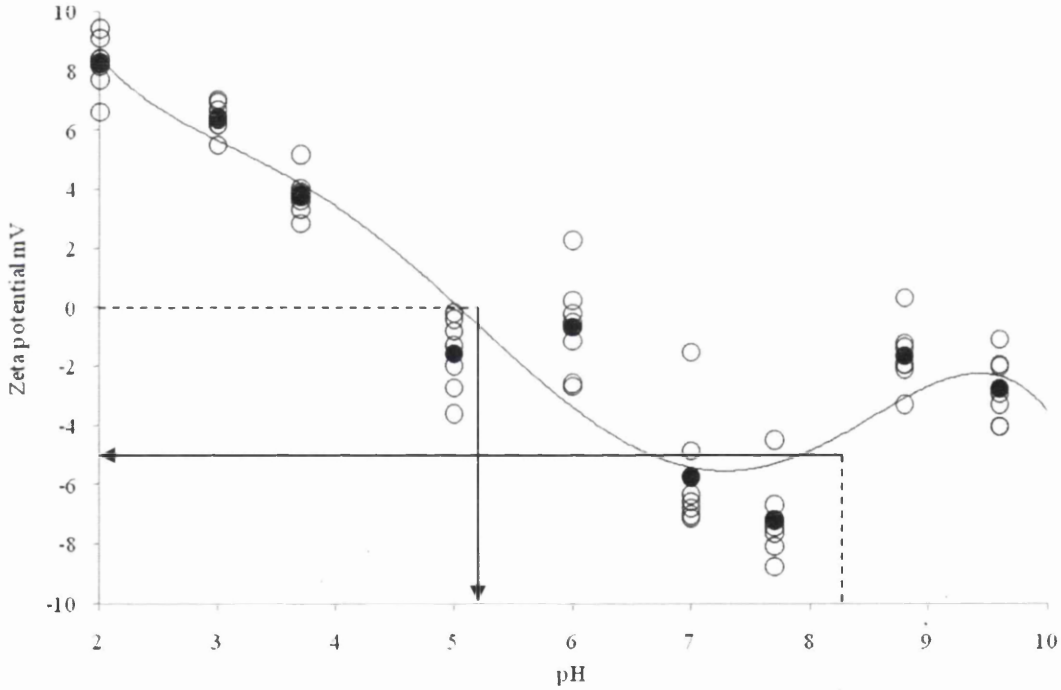

Figure 41: Combined zeta potential (ζ) plot of faeces from European sea bass fed Phase 2 experimental feeds. Trend line is fitted to the mean values, denoted by filled circles. The position of the combined isoelectric point (IEP) is shown, and is calculated as pH 5.01. The ζ at pH 8.05 was calculated as -5.89 for the combined data.

ζ and IEP at the system operating pH of 8.05 are shown for each feed in Table 19. Data for both parameters were normally distributed, but no statistically significant differences were observed among feed groups for either parameter.

Table 19: Zeta potential (ζ) in millivolts (mV) at rearing system pH (pH 8.05) and isoelectric point (IEP) for faeces produced by European sea bass fed Phase 2 experimental feeds. Values are mean ± standard deviation. No statistically significant differences were noted ($p > 0.05$).

	ζ @ pH 8.05	IEP
Fish meal	-4.68 ± 1.51	5.30 ± 0.41
20 soy	-6.58 ± 2.12	5.29 ± 0.48
20 soy + blood	-7.03 ± 3.08	4.92 ± 0.89
35 soy	-4.97 ± 2.31	5.40 ± 0.57
35 soy + blood	-5.07 ± 0.85	4.72 ± 0.19
20 wheat gluten	-5.23 ± 2.74	5.39 ± 0.76
20 wheat gluten + blood	-3.43 ± 0.28	5.18 ± 0.43

Regression of ζ at pH 8.05 against the particle size distribution kurtosis showed that there was no statistically significant relationship between the parameters ($F_{1, 19} = 0.283$, $r^2 = 0.015$, $p > 0.05$) or between ζ and particle size distribution skewness ($F_{1, 19} = 0.152$, $r^2 = 0.008$, $p > 0.05$).

Calculated faecal production

It was not possible to determine faecal production due to the FCR not being recorded in this experiment.

Faeces settling velocity

The mean settling velocity of European sea bass faecal particles calculated from particle size and density was 2.7 cm s^{-1} (overall mean for all replicates from all Phase 2 feeds, as no statistically significant differences were found among feeds). This value was then used to determine the time taken to settle in 10 cm of water, as detailed in Chapter 2, Section 2.16.

The settlement data are presented in two plots, split to allow better resolution at the lower end of the x axis, the first (Fig. 42) representing 10 to 70% of the distribution, and the second (Fig. 43) being 70 to 95% of the distribution. The data for the 0 to 10% fraction of the particle size distribution is excluded from the dataset due to this

being made up of the coarsest particles in the distribution and as such they would be expected to settle out of suspension almost instantaneously. The 95 to 100% fraction of the distribution by contrast would be expected to remain in suspension indefinitely, due to their being the finest particles in the distribution, and as such are also excluded from the dataset. The data for the settlement time of faeces at both 50% and 90% fraction of distribution were found to be non-normally distributed, and were tested non-parametrically as no transformation was possible.

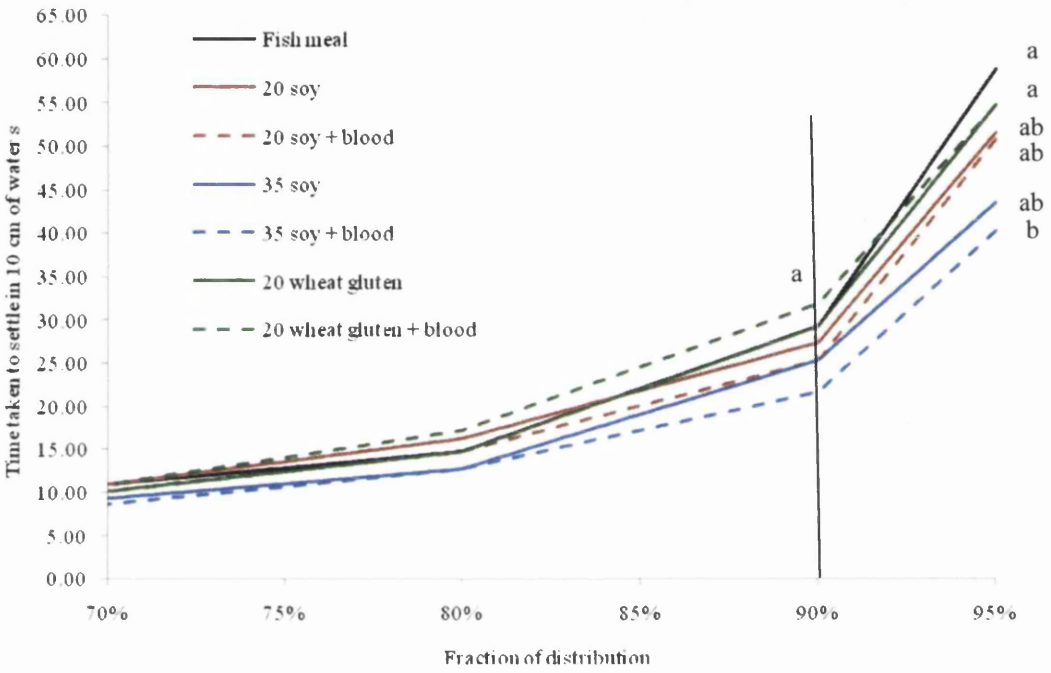

Figure 42: Calculated time taken (s) for European sea bass faecal particles in the range 10 to 70% of the total particle size distribution (from coarse to fine particles) to settle in 10 cm of water. Different letters signify statistically significant differences in the time taken for 90% of the distribution to settle ($p < 0.05$).

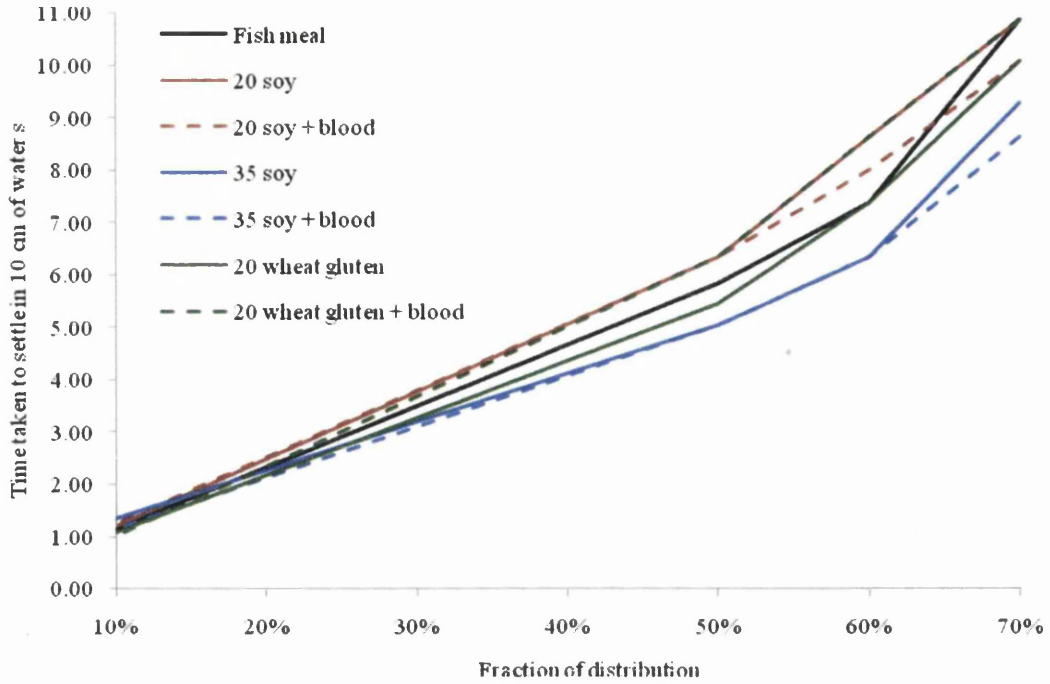

Figure 43: Calculated time taken (s) for European sea bass faecal particles in the range 70 to 90% of the total particle size distribution (from coarse to fine particles) to settle in 10 cm of water. No statistically significant differences were noted in the time taken for 50% of the distribution to settle ($p > 0.05$).

Statistically significant differences were noted in the 50% data set. The 20 wheat gluten + blood feed yielded faeces with significantly slower settling rates than for the 35 soy + blood feed. This in turn settled significantly more rapidly than the fish meal and both wheat gluten feeds.

Faecal rheological characteristics

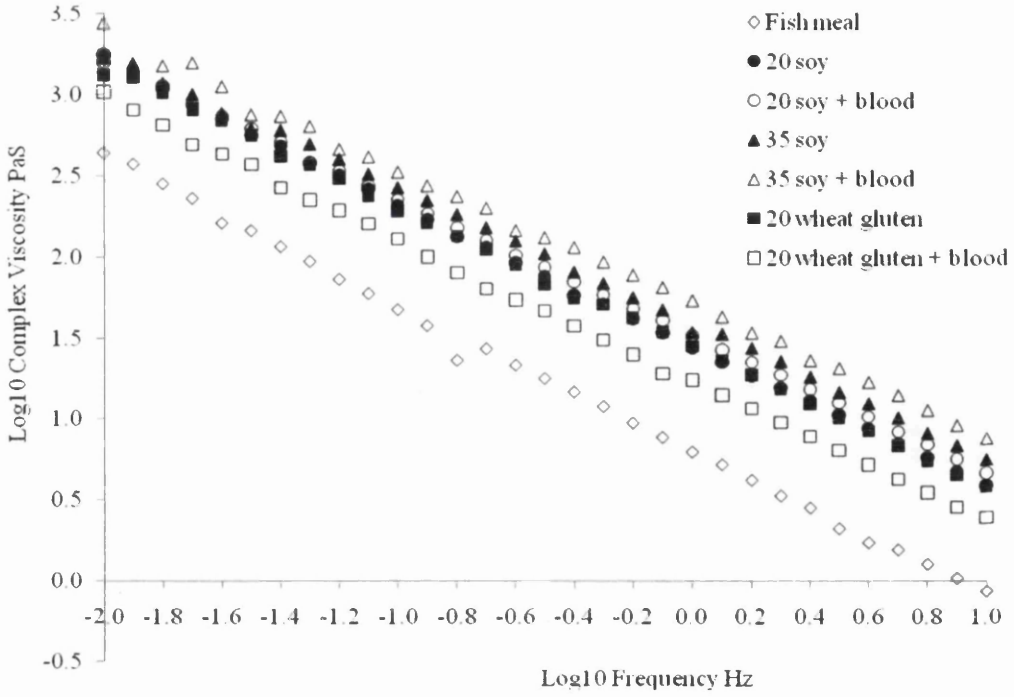

Figure 44: Frequency sweep data from the faeces of European sea bass fed the Phase 2 feeds.

The rheological data were analysed by plotting the $\log_{10}$ complex viscosity (Pa.S), the main effect, against $\log_{10}$ frequency (Hz) derived from the frequency sweep analysis (Chapter 2, Section 2.17) and were analysed as per Chapter 2, Section 2.18. Variances were found to be equal ($p > 0.05$), and statistically significant differences were recorded between feed treatments ($p < 0.0001$) for the main effect (Fig. 44). There was however also a significant interaction term (tank * feed, $p < 0.0001$) indicating violation of assumption of homogeneity of regression slopes. Due to this the model is deemed to be unsound in this case as there are differences within tanks exposed to the same experimental feed.

Table 20: Mean viscosity (Pa.S ± standard deviation) of faecal material produced by European sea bass fed the Phase 2 experimental feeds.

	Mean viscosity Pa.S
Fish meal	72.4 ± 55.2
20 soy	291.2 ± 247.4
20 soy + blood	293.4 ± 89.9
35 soy	325.8 ± 222.6
35 soy + blood	435 ± 127.8
20 wheat gluten	262.1 ± 56.5
20 wheat gluten + blood	172 ± 167.4

The mean viscosity of faecal material is shown in Table 20. The data were found to be normally distributed, but showed no statistically significant differences among treatments due to the high standard deviations seen. As viscosity is an analogue of faecal stability, the trend from least to most stable as determined from Figure 44 and Table 20 is as follows:

> Fish meal < 20 wheat gluten + blood < 20 wheat gluten < 20 soy < 20 soy + blood < 35 soy < 35 soy + blood

The fact that statistically significant differences were measured in the dynamic data (Fig. 44) supports the assumptions made from the visual analysis i.e. the stability ranking, although pairwise post hoc analysis is not possible due to the violation of the assumption of homogeneity of regression slopes.

The storage or elastic modulus (G') and loss or viscous modulus (G'') data demonstrates that the inclusion of plant proteins increased both G' and G'' over the values obtained using fish meal (Fig. 45). The greatest increase was seen with 35 soy + blood where both increased by an order of magnitude, while 20 wheat gluten + blood demonstrated the lowest increase over fish meal. This demonstrates that the addition of plant proteins results in faeces which are more viscous and have a greater elastic component, i.e. they are more solid like, and so more resistant to shear forces.

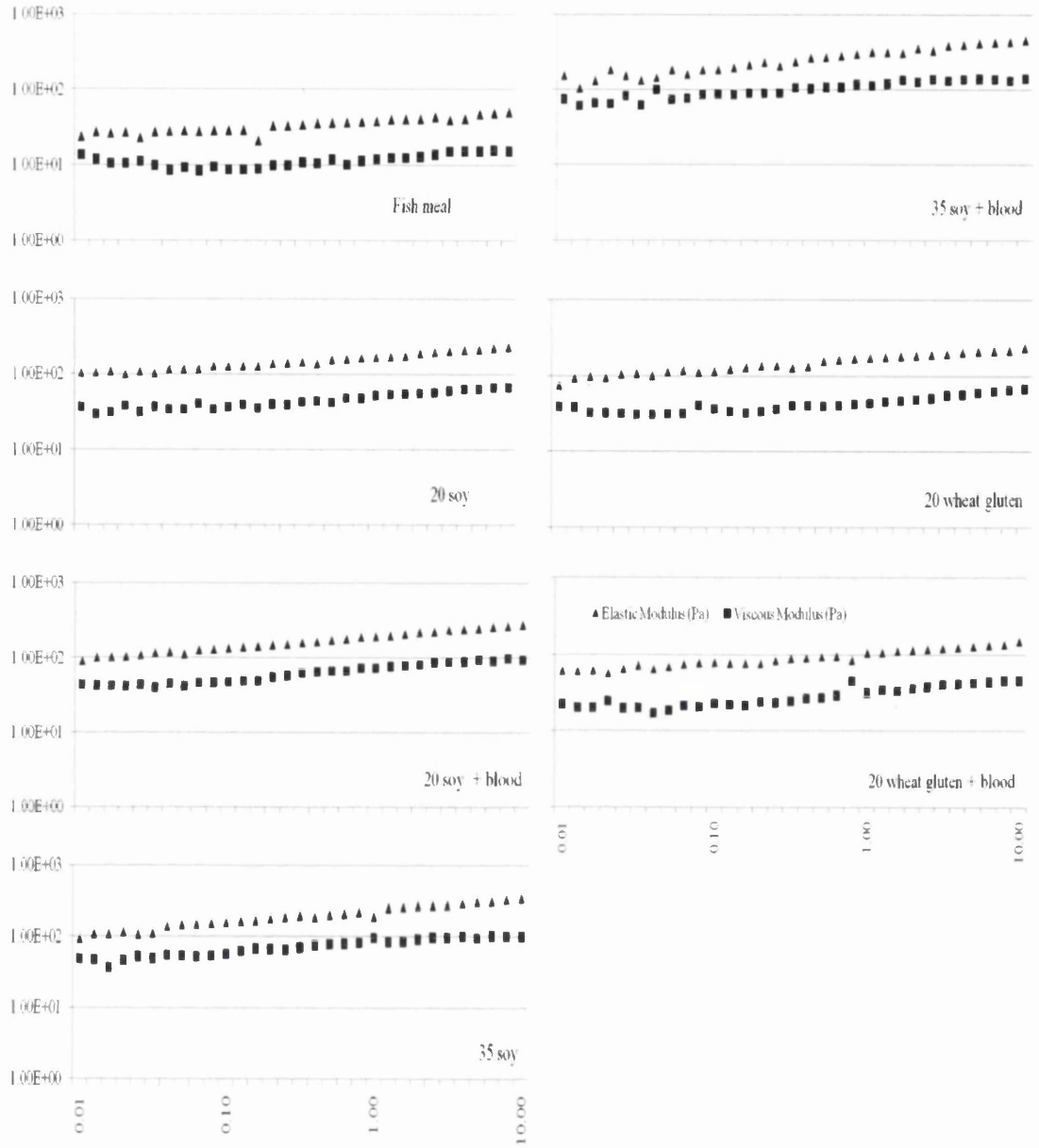

Figure 45: The storage modulus (elastic modulus, G') and loss modulus (viscous modulus, G'') as a function of oscillatory frequency, for the faeces of European sea bass fed Phase 2 experimental feeds.

No statistical analysis was identified which could satisfactorily test the data for statistical significance of differences, and thus no analysis was undertaken but the known relationships described. All plots also show the G'' to be higher in value than the G', showing that all fish faeces irrespective of protein source have a more prominent elastic behaviour than viscous behaviour.

4.4 Discussion

The objectives of the Phase 2 experiments, having identified the most suitable plant protein sources to take forward from Phase 1, were to investigate the feasibility of increasing plant protein inclusion levels, to carry out more detailed examination of the physical properties of fish faeces, and to identify and test a potential flocculant feed additive (blood meal). The results for fish growth and feed digestibility were comparable to those in Phase 1 experiments for diets of similar composition (i.e. 25% inclusion of wheat gluten or HiPro soya in Phase 1 versus 20% inclusion in Phase 2). Furthermore, no detrimental effects of increasing the inclusion level of HiPro soya to 35% were observed. Such results at higher inclusion levels have been recorded for other species of fish, with the notable exception of salmonids, by previous authors as referenced in Chapter 3, Section 3.4. Based on the combined Phase 1 and Phase 2 findings, soybean meal can be safely incorporated at up to 35% and wheat gluten at 25% substitution of fish meal by weight for either *Gadus morhua* or *Dicentrarchus labrax,* without significant detriment in growth performance.

In regards to faecal physical properties, the particle size distributions recorded in the Phase 2 experiments fell into similar ranges as in Phase 1. However, it is notable that the two smaller peaks observed in Phase 1 were replaced in the Phase 2 profiles by one peak at around 91 μm, possibly due to having selected different particle size groupings in the instrument. The positions of the peaks in Phase 2 are very similar between species, with sea bass exhibiting a slight shift at the coarsest end of the scale to a larger particle size. The two wheat gluten feeds plus fish meal yielded different particle size distribution patterns compared to the rest of the feeds, their peaks being shifted vertically in the fine and coarse regions of the particle size distribution. However, the pattern is reversed when compared to the findings of the Phase 1 experiments, with the first two peaks being shorter and the latter peak being taller in both species, suggesting that plant proteins, apart from wheat gluten, result in *finer* particles than fish meal. The reasons for the differences noted between the particle size distributions between Phase 1 and Phase 2 are unclear, but could potentially be due to differences in the batches of feeds produced for the each experiment. It is important to remember it is impossible to determine whether differences between

peaks are significant, and therefore the skewness data, as discussed subsequently, is used to determine the differences between particle size distributions.

Cod faeces were clearly divided into two classes of skewness, with fish meal and both wheat gluten feeds being coarse skewed and the remaining feeds being strongly skewed towards fine particles (Fernandes and Tett, 2001). Sea bass faeces ranged from fine skewed to strongly skewed towards fine particles, with fish meal having a significantly lower value than the majority of the soy based diets. These analyses confirm the findings from the visual analysis of the particle size distribution plots, in particular in the case of cod where the differences are pronounced, despite statistical power being too low for full analysis. The generally greater values of skewness (finer particles) for sea bass in comparison to cod supports the supposition made in Chapter 3 that there is more physical and chemical processing of the feeds in the GI tract of sea bass. It should be noted at this point that published literature also shows sea bass faecal particles to have a smaller mean diameter than those of cod, at 1.12 mm (Magill *et al.*, 2006) and 1.6 mm (Cromey *et al.*, 2009) respectively. The supposition of greater GI tract processing is further supported by the higher protein digestibility measured for sea bass compared to cod. Interestingly, the same skewness versus kurtosis relationship seen in Phase 1 was replicated here. Determination of the underlying mechanisms at work in the GI tracts of these animals may go some way to explaining these phenomena.

The trends for volume of faecal production, ζ, and faecal particle settling times were broadly similar to those found in the earlier Phase 1 experiments. Increasing the inclusion level of HiPro soya did not have any effect on the quantity of faeces produced. In terms of ζ, the cod data did show a weakly negative but statistically significant relationship between ζ and particle size distribution skewness, so that as ζ became more negative (greater repulsion between particles/ greater instability), so the value of skewness increased (particles become finer). This relationship had been expected for both fish species during Phase 1, as while ζ decreases so the electrostatic repulsion between particles increases reducing the likelihood of flocculation and an increase in particle size (Li *et al.*, 2008a). In terms of settling times, increasing the inclusion level of soybean meal from 20% to 35% resulted in more rapidly settling faeces for both fish species.

The effects of including blood meal in the feeds at a level of 5% inclusion by weight were limited, being associated only with slower faecal settling times for cod receiving 20% wheat gluten. It is possible that there is an interaction between blood meal and wheat gluten in the GI tract of the cod resulting in a slight shift of the particle size distribution to the fine end of the scale. If this was a physical interaction between these two feed ingredients, then it would be expected to be seen also in faeces from sea bass. On close inspection of the sea bass particle size distribution and skewness data, there is a small (non significant) trend indicating the same effect. It may be possible therefore that indeed blood meal and wheat gluten do interact to some extent in the GI tract, resulting in finer particles. This aspect deserves further research, as confirmation of such an interaction would be counter to the desired effect of blood meal addition. The concept of adding blood meal as a material to exploit the charge characteristics of the suspended faecal particles was therefore not successful in increasing particle size distributions, settling rates or rheological stability (= ability of a material of resist shear forces and breakup cf. suspension stability) of the faecal matter for either fish species

The absence of an effect of blood meal in Phase 2 experiments may be in some part explained by processing of the haemoglobin in the GI tract, i.e. by the acidic environment found in the stomach releasing ferric iron from its ingested matrix. The processes at work controlling iron uptake in the GI tract of fish are not well defined (Bury *et al.*, 2003), but it would be expected that ferric reductase (although poorly described in fish), would reduce ferric iron to ferrous iron. Ferric iron could also be affected by reducing agents in the feed, for example, ascorbate (Raja *et al.*, 1992). The overall result would be a decrease in ferric iron and a possible increase in the ferrous iron present in the GI tract, should some of it not be absorbed. It is also known that the intestine of marine fish secretes large quantities of bicarbonate, resulting in the precipitation of divalent cations (Walsh *et al.*, 1991) including ferrous iron, as $Fe(HCO_3)_2$, which may be expected to make this unavailable for any subsequent particle interactions. Ferrous iron has also shown to be preferentially absorbed by European flounder *Platichthys flesus* (Bury *et al.*, 2001), whereas ferric iron has been shown to be poorly absorbed in relation to ferrous iron in chicks (Wood *et al.*, 1978).

All this information leads to a possible conclusion that the addition of iron as haemoglobin in the feeds for marine fish, despite being known to work as a flocculant for post-production treatment of effluent water (Adin *et al.*, 1998, Zhou *et al.*, 2008) may not have the desired effect due to the digestive processes it is subjected to in the GI tract. It is also possible that the blood meal production process and/or its extrusion during feed production may alter the availability or iron to the fish or as a flocculant. However, there is conflicting evidence on the effects of blood processing, with some authors reporting increased bioavailability of Fe with heat and pressure application (Wood *et al.*, 1978) and thus potentially a decrease in Fe available for flocculation; some reporting no change in Fe bioavailability (Pinto *et al.*, 1997); and some finding that Fe becomes less bioavailable post processing due to the liberation of iron from the haem molecule (Kristensen and Purslow, 2001) and thus potentially leading to an increase in egested Fe.

The iron content of dried swine blood is 2,273 to 2,700 mg Fe kg^{-1} (Hertrampf and Piedad-Pascual, 2003, Cromwell, 2006). Using these values it can be calculated that the fish are consuming 114 to 135 mg Fe kg^{-1} dry feed consumed (e.g. 2700 mg Fe kg^{-1} blood x 5% inclusion). Carriquiriborde *et al.*, (2004) added 175 mg Fe kg^{-1} feed in a study into iron absorption in *O. mykiss* and reported faecal Fe of 12.3 µmol g^{-1} d.m. This value equates to 0.7 mg Fe g^{-1} dry faeces (0.0000123 mol g^{-1} d.m. Fe x 55.8452 = 0.0007 g g^{-1} d.m), and assuming faecal moisture content of 90%, equates to 0.07 mg Fe g^{-1} faeces (70 mg Fe kg^{-1} faeces) as produced. This would clearly be greatly diluted in the effluent water. Previous authors investigating Fe-based flocculation in municipal effluent water found optimal dosage of Fe as added to the effluent flow to be between 20 and 40 mg l^{-1} when using $FeCl_3$ (Adin *et al.*, 1998, Prakash *et al.*, 2007) or 18 mg l^{-1} when using $FeSO_4$ (Prakash *et al.*, 2007). Prakash *et al.* (2007) were using effluents containing 65 to 100 mg l^{-1} of total suspended solids. It therefore follows that the amount of Fe excreted in the faeces was likely well below the optimal dosage in these situations. Also, if the Fe is egested in the faeces as biomineralised $Fe(HCO_3)_2$ as speculated in the previous paragraph, then it may not be available as a flocculant. Further work is recommended to elucidate the Fe species in the finished feeds and Fe egested after the consumption of blood meal, and to determine a more suitable feed additive which will remain unchanged by the gut processes while not affecting growth or fish health.

The data for viscosity of sea bass faeces fits very well with the published literature, with the fish meal feed displaying an average viscosity of 72.4 Pa.S, with the plant protein treated feeds ranging from 172.0 to 435.1 Pa.S. Brinker (2007, 2008) reported an average viscosity of faeces for fish meal-fed trout as 72.4 Pa.S, increasing after binder addition to between 182.0 and 265.1 Pa.S (comparable sized fish to those used in this study). The results of the Phase 2 experiments indicate that plant protein inclusion can increase the viscosity of faecal material in marine finfish comparably to addition of non-starch polysaccharides (such as guar gum) for rainbow trout, albeit that high variability among viscosity readings was problematic in the current study, especially for cod. Furthermore, a statistically significant interaction term demonstrated that there were masked effects in the data, suggesting differences between tanks within feed treatments. Such differences could be introduced to the data via environmental or fitness effects introducing variability and through differences in sample processing, despite the best efforts of replication, randomisation and standardisation in the experimental design. It was important to check for such differences by testing the homogeneity of regression slopes, as a simple ANOVA would have shown significant differences between feeds while the model would have been unsafe.

The storage and loss moduli (G' and G'') show that almost all the faecal samples for both fish species show near solid-like behaviour with the storage modulus G' being greater than the loss modulus G''. That is, the elastic or solid part dominates the rheological properties of these samples (Chen *et al.*, 2005). This data backs up the findings in relation to viscosity, whereby the addition of plant proteins results in a more solid like waste, with a greater resistance to disruption through shear forces, i.e. it is more rheologically stable. However, the expected increase in particle size with rheological stability as reported by Brinker *et al.* (2007, 2008) was not observed for either species in the current research. In fact, it was seen that the least stable faeces (fish meal and wheat gluten feeds) resulted in the finest particles in the particle size distributions. This was also a reversal of the trends seen in Phase 1 in relation to these feeds.

The clear differences observed between the sea bass and the cod, which do not appear to be due to material differences, and the considerable variation despite the

use of near identical methods, suggests that there are issues with the rheological analysis in these experiments. It is possible that the level of pre-analytical processing, such as freezing and filtering, could have altered the physical makeup of the material. In order to surmount such difficulties, it would be desirable in future to analyse fresh whole faecal matter. The first obstacle in the way of this was the limited availability of material., with multiple stripping events and pooling of samples required even to obtain sufficient quantities of material for the analyses undertaken. A lack of prior knowledge of the amounts of faeces required for the various analyses carried out and of the amounts of faeces that would be produced by the fish was one reason for this lack of material. This could be rectified in future by using greater numbers of fish per replicate group or using larger individuals. As this research was licensed by the UK Home Office, there was the requirement to comply with regulations on minimising the number of animals used in the research. Finally, the logistical and financial practicalities of obtaining large fish for the purpose of high faecal production meant that these were not available to us for the purposes of this research.

The second potential introduction of error into the data was the need to filter out the largest particles from the faeces, due the requirement for the gap in the rheometer to be at least 10 x the diameter of the largest particles. As gap width for standard analyses would normally be no greater than 1 mm, the presence of particles of over 100 μm would ideally be avoided. In this case it was decided to filter the material at the 250 μm level, thereby removing the largest particles but retaining as much of the faeces as possible. However, it should be possible in future to analyse the material without the need for filtration, for example by using different rheometer geometries such as vane geometries. The potential for this type of rheometry then comes back to the availability of material, as large quantities (> 10 ml) would be required for this method. Nonetheless, the data obtained does strongly indicate that plant proteins improve the rheological stability of European sea bass faeces, and further optimisation and refinement of the analytical methods is recommended to fully explore this area of faecal characterisation and feed formulation.

4.5 Conclusions and considerations for future research

In conclusion, the Phase 2 experiments have shown that HiPro soya can safely be included at 35% substitution of fish meal by weight and confirms that wheat gluten can safely be included at 20% substitution of fish meal by weight for either *Gadus morhua* or *Dicentrarchus labrax,* without significant changes in growth performance. As in the Phase 1 studies, wheat gluten would be the most suitable candidate as a fish meal replacer, due its high digestibility and reduced faecal output per unit of fish growth, resulting in reduced output in the fine particle size ranges in cod. Production in the case of sea bass was not calculated. The substitution of fish meal by HiPro soy, at both inclusion levels, in general resulted in undesirable faecal production trends, including in the fine particulate ranges. The addition of blood meal as a coagulant to enhance the inherent electrical instability of fish faecal material, as discussed in Chapter 3, section 3.5, did not impart any measurable effects on the faecal material.

The determination of the rheological properties of marine fish faeces was moderately successful. The sea bass data demonstrated a pattern in rheological stability which appears to go against the information gained from the particle size distribution and skewness data and the findings reported from the literature, in that the more stable faeces resulted in finer particle sizes. While good progress was made, certain technical problems were not surmounted within the timeframe and available resources of the project, such as the optimal rheometer cell geometry to use, the volume of material available, and how to analyse intact faeces (complete with large particles), and further work is required in this area to reduce the high variability observed.

In terms of faecal settling information, relatively simple procedures could be carried out in future experiments to enhance the usability of this information. Assuming enough faecal material is available (the limiting factor in this research for many analyses) relatively simple desktop experiments could be carried out, such as simple jar experiments to determine settling times as carried out by Prakash *et al.* (2007) and Li *et al.* (2008a) with demonstrable success.

Further work is also recommended on the fate of the iron contained within blood meal following passage throughout the GI tract of marine fish, to elucidate why no effects of blood meal inclusion were observed. Also, as discussed previously, the suggested interaction of wheat gluten and blood meal observed during Phase 2 deserves further research to determine whether this is a reproducible phenomenon. Finally, further research should be carried out on other potential flocculants which could be added to feeds for farmed fish to enhance the required characteristics of the faeces.

To summarise, specific questions raised by the current research that are desirable to investigate in future are:

- How can the rheological analysis of fish faeces be refined to enable reproducible and representative information to be obtained, i.e. using whole faeces with limited pre-processing?

- What are the Fe species egested following digestion of blood meal, and why do these not affect the particle size of the effluent as expected?

- Which materials might be more suitable as feed additives than blood meal in acting as a flocculant in the faeces, while ensuring that good growth rates are maintained, and what are the effects of these additives on the physical properties of the faecal material?

Chapter 5

General Discussion

5.1 Overview

The research forming this thesis aimed to determine the effects of substituting fish meal with plant proteins on the growth and faeces characteristics of Atlantic cod and European sea bass, combining established experimental methods for aquaculture feed evaluation with engineering-based particle and fluid analyses. This was undertaken to provide greater knowledge of the effects of plant proteins in formulated fish feeds on effluent management in closed or semi-enclosed culture systems, and on effluent dispersal in open (cage-based) rearing systems.

The research findings showed that the four tested plant protein sources, those being high protein soybean meal (HiPro soya), low protein soybean meal (LoPro soya), wheat gluten and lupin meal, were suitable as fish meal replacers for Atlantic cod. Additionally, these plus two additional sources (dehulled faba beans and whole faba beans) were also suitable for European sea bass up to 25% inclusion by weight. Additionally, HiPro soya was found to be acceptable at up to 35% inclusion for both fish species. Based on volume of faecal production and physical properties of the faeces, wheat gluten was identified as the most suitable plant protein source for use in feeds for either fish species in closed or semi-closed culture systems incorporating physical filtration systems. This is due to the overall reduction in fine particulates expected as a result of reduced faecal production. Most of the other tested plant proteins resulted in more rapidly settling faeces, and as such were suggested to be more suitable for systems incorporating an effluent settlement stage, or in open water situations in which rapid settlement was required.

Particle size distribution, zeta potential and rheological stability were tested as tools for characterising faecal material, and in determining the effects of dietary plant proteins. The routes by which such parameters are expected to provide useful information for aquaculture system management are summarised in Figure 46. The research has shown that there are measurable differences between faecal wastes in these physical characteristics following the substitution of fish meal by plant proteins, while also highlighting scope for further method refinement. By simply determining the anticipated volume or mass of faeces through conventional feed digestibility studies, information is lacking on what may happen once that faecal

waste is suspended in the rearing system or surrounding water and how any effluent treatment system may deal with the waste. Similarly, by investigating the effects of binders or coagulants on the faecal characteristics in isolation without knowledge of total faecal production, essential information as to the potential of such materials for inclusion in commercial feeds would be missing. The current research has attempted to combine these experimental approaches to provide added value.

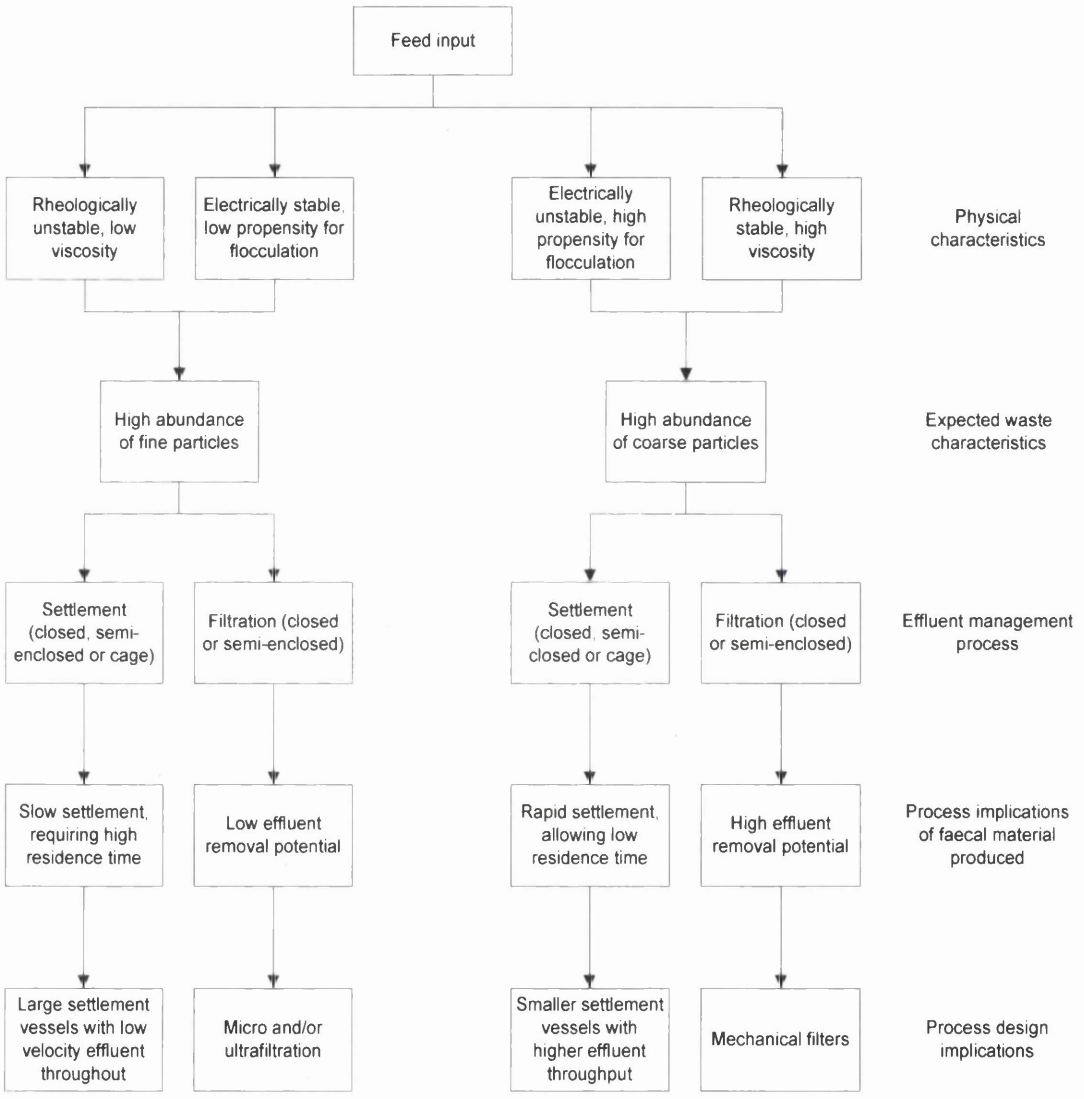

Figure 46: Flow diagram of expected implications of the physical characteristics of faeces in fish culture systems.

By employing the methods shown here, it is possible to predict not only how much waste will be produced by a given fish species on a given feed, but also what the physical characteristics of that waste will be. This could then be applied to tailor the

filtration and effluent treatment system to the fish species and feed type in question, or conversely to match fish and feeds to systems to minimise effluent production. Furthermore, the potential to alter the charge distribution on faecal particles may allow the proportion of fine particles to be reduced for any feed. This would not necessarily affect the absolute mass of waste, but may aid in its removal in closed or partially-closed culture systems through larger particle sizes and more rapid settling, the latter also being of potential importance in open water systems.

Particle size distribution, from which the settling properties and faecal production information have been determined in this research, is determined in part by the charge on the particles and the stability of the faecal material. Therefore it is important to discuss the importance of these physical characteristics and their application to aquaculture effluent management.

5.2 Zeta potential and suspension destabilisation

Zeta potential analysis showed that faecal matter from Atlantic cod and European sea bass had a moderately negative charge at culture conditions, suggesting a moderately stable suspension. The addition of blood meal with the aim of increasing the instability of the suspension and encouraging particle flocculation to increase particle size, effluent removal potential and settlement rate did not have the desired effect. This, as discussed in detail in Chapter 4, is thought to be due to digestive and biomineralisation processes in the gastrointestinal tract. Nonetheless, there does appear to be considerable potential for the use of zeta potential in investigating flocculation properties of faeces with different additives.

Suggestions for feed additives are made below, and while these are by no means exhaustive, they do illustrate the potential for the addition of materials which do not physically stabilise the faeces in a rheological sense, but *de*stabilize the effluent in an electrical, zeta potential, sense. Two of the main routes for zeta potential to be exploited are coagulation though surface charge neutralisation and polymer bridging (Hjorth *et al.*, 2008) illustrated in Figure 47. The first of these is the mechanism by which iron causes flocculation for treatment of effluent waters, while the second is

perhaps the most promising for aquaculture applications, in the light of current research findings.

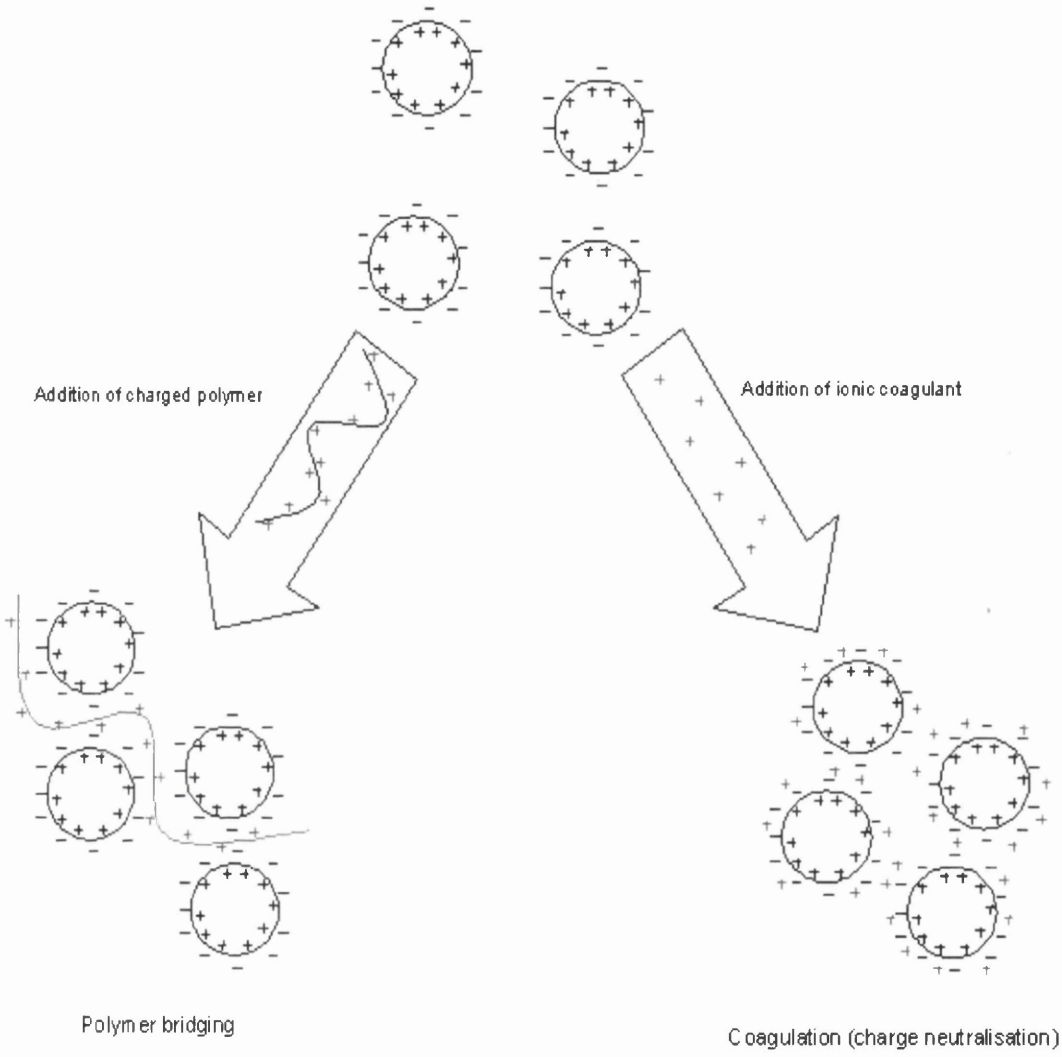

Figure 47: Schematic of the two main effects of charged materials present in faeces, polymer bridging and coagulation through charge neutralisation.

Chitin and chitosan are positively charged and readily available materials, being a by-product of crustacean processing, with a charge density dependent on pH and the degree of deacetylation. These charge properties make chitosan an effective bioadhesive, though the process of charge neutralisation, readily binding to negatively charged surfaces such as mucosal membranes, a property which could make it a useful flocculant (Li *et al.*, 2008b). It has also been shown to not be utilised to any major extent by *S. salar* (Olsen *et al.*, 2006), suggesting it may pass through the animal into the faeces and resultant effluent.

Polyacrylamide has been proved as a good coagulant, especially in its branched form, in swine manure, added to the manure at 0.6 g kg^{-1}, removing up to 95% phosphorus via polymer bridging (Hjorth *et al.*, 2008), resulting in highly resistant compact flocs. It may be possible to use a material with similar charge properties to polyacrylamide in feeds for farmed fish. However, despite this material in particular being shown to be not significantly toxic, with 5% inclusion not having any significant adverse effects in rats and dogs, note should be taken of the very small amounts of the toxic compound acrylamide as used in the production of polyacrylamide, which may be a cause for concern in the environment at very high levels (Andersen, 2005).

Finally, it may also be possible to engineer, to some extent, the production of extracellular biopolymeric flocculants by bacteria, fungi, yeasts and algae. De Schryver *et al.* (2008) suggested that selecting for a start-up inoculum in wastewater with co-aggregative species to promote the overall aggregation efficiency of the microbial biomass (negative charge of -20 to -30 mV) may be of interest. This is discussed in terms of treating effluent post production, but it may be possible that such a route could be used in the design of 'probiotic' feed additives, encouraging the production of biopolymeric flocculants in the gastrointestinal tract of the fish.

Zeta potential as introduced in this work has only rarely been employed in the characterisation of aquaculture wastes, despite being commonly used in the analysis of municipal waste water and sludges. Authors have successfully used the analysis of zeta potential in determining the mechanisms at work in flocculation of municipal wastewater (Zhang *et al.*, 2007b) and in determining the coagulation potential of flocculants municipal sludges (Kim *et al.*, 1999, Zhang *et al.*, 2007b, Thapa *et al.*, 2009, Zouboulis and Tzoupanos, 2009). It has also been used in demonstrating the effects of charge on floc strength (Kara *et al.*, 2008) and in assessing the fouling potential of effluents though determining the stability of the effluent and its correlation with fouling (Schrader *et al.*, 2005). These studies demonstrate the potential of the zeta potential methodology in analysing aquaculture effluent flocculation potential, the effects of flocculant addition, and the impact of such activities on effluent stability and wastewater filtration efficiency.

5.3 Rheology and implications for plant protein substitution

The addition of plant proteins into the feeds for Atlantic cod and European sea bass resulted in a general increase in the viscosity and in the mechanical stability of the faeces over feeds containing purely fish meal as the protein source. The manipulation of faecal viscosity and rheological stability of fish waste has previously been examined by the addition of non-starch polysaccharides, such as guar gum. It appears that there may be optimal inclusion levels of non-starch polysaccharides, as Brinker (2005b, 2005d, 2007, 2008) reported higher viscosity and resistance to shear resulting in a higher effluent removal potential at 0.3% inclusion of guar gum. Amirkolaie *et al.* (2005) on the other hand reported finer particle production possibly due to less resistance to shearing, leading to reduced removal efficiency, at 8% gum inclusion. Also, digestibility of feeds shows considerable variation among authors, as discussed in Chapter 1, with several authors reporting reduced digestibility with the addition of guar gum (Fagbenro and Jauncey, 1995, Amirkolaie *et al.*, 2005, Leenhouwers *et al.*, 2006) while others reported no influence on digestibility (Leenhouwers *et al.*, 2004, Brinker *et al.*, 2005b).

A further potential candidate to increase digesta viscosity is carboxymethylcellulose. However, previous studies evaluating it as a potential binder have shown that at 5% it can inhibit proteolytic enzyme activity in the digestive tracts of *P. olivaceus* (Yamamoto and Akiyama, 1995). In the tongue sole, *Cynoglossus semilaevis*, it can also can result in poor growth performance when compared to sodium alginate (Liu *et al.*, 2008), possibly for the same reason. Therefore, it appears that increasing the viscosity of the chyme can affect both effluent removal potential and feed digestibility, depending on inclusion level, and further work will be required to quantify this. It should also be borne in mind that including indigestible feedstuffs e.g. dietary bulk agents, while increasing faecal firmness, will increase the production of faeces significantly (Adin *et al.*, 1998).

The current research coupled with past work by, most notably, Brinker (2005b, 2007, 2008), has shown that the rheological analysis of aquaculture wastes can be a useful tool in their characterisation and therefore in feed design. However, there appears to be much work required to enable to the methodology to be fully repeatable given the

different physical make up of the wastes from different species. It is envisaged that, should a simple bench top procedure be identified, rheological analysis might play a significant part in waste analysis in the future.

5.4 Impact of physical characteristics in aquaculture facilities

Due to the range of different fish culture systems used in the aquaculture industry, only a broad overview, based on current research findings, is presented below of the effects of effluent characteristics on different rearing system. Table 21 outlines the broad classifications of culture system and how effluent characteristics may affect each of these, considering all potential farming conditions and what are the desirable and undesirable particulate characteristic in each case, and how might these be encouraged or mitigated against. The systems considered are cage farms with locally high impact and no impact over the wider area, or low impact over a large area; land-based flow through operations, and land-based farms in which settlement or filtration is the major effluent treatment process. This table provides an overview of the basic effects of different faecal characteristics and their impact on the operation of effluent treatment systems and/or environment impacts.

Table 21: Overview of characteristics of fish faeces, potential associated problems leading to negative impacts on the aquaculture operation, the solutions available and the methods by which these solutions may be achieved.

	Culture system or main effluent treatment mechanism				
	Cage (high local / no wide impact	Cage (low local / low wide impact)	Flow though	Settlement	Filtration
Critical faecal characteristic	Settlement (as a function of particle size)	Settlement (as a function of particle size)	Production	Settlement (as a function of particle size)	Particle size
Problem	Slow settling	Rapid settling	High production	Slow settling	Fine particles
Solution	Encourage flocculation, increase rheological stability	Encourage electrical stability, reduce rheological stability	Increase digestibility	Encourage flocculation, increase rheological stability	Encourage flocculation, increase rheological stability
Method	Addition of flocculant/ binder	Select most suitable feed	Reduce indigestible fraction	Addition of flocculant/ binder	Addition of flocculant/ binder

5.5 Future work and concluding remarks

In conclusion, it has been shown that European sea bass and Atlantic cod are receptive to a range of plant protein sources as fish meal replacers at moderate inclusion levels. It has also been demonstrated that there is considerable potential for the use of a range of analytical methods in determining the physical properties of faecal waste. These physical properties are measurably affected by plant protein inclusion, resulting in faecal waste which may be more or less suited to any particular form of aquaculture, depending on these properties.

A range of potential specific future directions for research has been suggested in discussing the results. The wider field however requires future work to be carried out in refining the analytical methods used in this research to a point where such information can be incorporated into waste production and/or dispersion models. This could allow faecal production and behaviour post-production to be taken into account when planning and licensing new aquaculture operations. In addition to this, it is recommended that the development of additives for aquaculture feeds designed to increase faecal stability and encourage flocculation of faecal particles be examined more fully. This will allow feeds to be designed with specific culture systems in mind, for example, the development of a feed resulting in low faecal production, high faecal stability and high flocculation potential for fish cultured in recirculating aquaculture systems. It would also allow additives to be identified which would bestow these properties on any feed when added at prescribed levels. It is believed that there is significant future potential in using the methods trialled in the research reported herein in reaching this goal for efficient and sustainable aquaculture operations.

It is hoped that this research has added value to the methods currently used to analyse faeces within the aquaculture feed development industry by introducing new methods, and highlighting shortcomings and areas of future direction for such analyses and in more established methods. It is envisioned that such methods developed and tested in pursuing this work would be utilised during the development of feeds and testing of ingredients of additives. Once feeds have been designed to impart the required physical properties on faeces and these properties have been

tested in the laboratory and in the field, further on-farm testing should not be required thereby not requiring further simplification and potential reduction of experimental integrity. It is hoped that further work in terms of feed inputs and waste outputs will remain foremost in the industry's future sustainable development. It is important to remember however that such development must not inhibit the ability of the industry to be competitive in the sea food marketplace, and as such cost benefit analysis must be undertaken as the research moves forward into the commercialisation of specially designed technical feeds.

6 Appendices

Appendix 1

System monitoring and record keeping

Ammonia (salicylate method, Hach Programs 342 N, Ammonia LR TNT). Two millilitres of sample water was added to one "AmVer™ Diluent Reagent Test 'N Tube™ for Low Range Ammonia Nitrogen" vial. Two millilitres of ammonia free water was added to another vial as a blank. One packet of ammonia salicylate reagent powder was then added to each, followed by one packet of ammonia cyanurate reagent powder. Each vial was then capped tightly and shaken until all powder was dissolved and left for 20 min. At the end of the time, the blank was placed into the colorimeter and the machine zeroed, followed by the sample vial. Results were displayed as mg/l NH_3-N.

Nitrite (diazotisation method, Hach Programs 345 N, Nitrite LR TNT). Five millilitres of sample water was added to one "Test 'N Tube NitriVer® 3 Nitrite" vial. The vial was then capped tightly and shaken until all powder was dissolved and left for 20 min. At the end of the time, an empty "Test 'N Tube™" vial was filled with 5.0 ml of sample. This blank was then placed in the colorimeter and the machine zeroed, followed by the sample vial. Results were displayed in mg/l NO_2-N.

Nitrate (Hach Programs 361 N, Nitrate HR AV). A "NitraVer 5 Nitrate AccuVac® Ampul" was filled from a beaker containing at least 40 ml of sample water and inverted 48 to 52 times for one min. It was then allowed to rest for 5 min. At the end of time, a round sample cell was filled with 10 ml of sample water. This blank was then placed in the colorimeter and the machine zeroed, followed by the sample ampule. Results were displayed in mg/l NO_3-N.

Appendix 2

Proximate analysis of feeds and fish

Amino acid profile (peptide bound and free). Samples of feeds (100 to 1000 mg) were ground and homogenised after freezing at -80°C, while faeces samples (1 g) were remained wet, and weighed into a 100 ml bottle.

Stage 1 - Oxidation – Five millilitres of an oxidation mixture (0.5 ml H_2O_2 + 4.5 ml formic acid-phenol solution) was added to the sample and mixed for 10 to 15 s using a magnetic stirrer. The bottle was then sealed and placed in an ice bath for 16 hr. After this time, 0.8 g of sodium metabisulphate was added and mixed for 10 to 15 s to decompose excess reagent, and then left for 15 min. Oxidation was not performed in histidine analysis.

Stage 2 - Hydrolysis - Twenty five millilitres of a hydrolysis solution (5 g phenol + 2.5 l pure water + 2.5 l HCl) was added to the bottles, which were loosely capped, and heated to 110°C for 24 hr (caps were tightened after 1 hr). Following this the bottles were cooled to room temperature.

In the case of feeds, 15 ml of an internal standard (0.4006 g norleucine + 100 ml HCl + pure water to make up to 1000 ml) was added and mixed for 10 to 15 s. Following this 125 ml citrate buffer (98 g sodium citrate + 4 l pure water + 5 g phenol + 25 ml thiodiethnol + 80 ml hydrochloric + pure water to make up to 5 l) and 17 ml 7.5 M NaOH was added and mixed for 10 to 15 s. The pH was then adjusted to 2.2 using NaOH. The solution was filtered through a 0.2 μm filter by centrifugation at 8000 rpm for 4 min, and transferred to high pressure liquid chromatography bottles. Internal standard was not used in the case of faeces analysis, and the process was picked up at the point of adding citrate buffer. Following filtration, the volume was measured, taking into account the volume of the magnet. The solution was then transferred to high pressure liquid chromatography bottles.

Analysis was by Biochrom 30 Amino Acid Autoanalyser (Biochrom Ltd, UK) using a Midas Autosampler (flowspek ag, Switzerland).

Protein (Kjeldahl). Samples of dry feed and faeces were ground and homogenized after freezing at -80°C, and were weighed on nitrogen free paper weighing boats to 4 decimal places using a Foss 2030 weighing terminal connected to a Foss Kjeltec 2400 Auto System incorporating a 2400 Analyser and 2460 Sampler (FOSS Analytical AB, Sweden). Weight of sample was approximately 0.4 g (standard for samples with an expected protein content of for than 30%).

The weight was sent automatically to the Kjeltec instrument. Both sample and weighing boat were transferred to a 250 ml digestion tube. Two blank samples of weighing boats only were also added to digestion tubes.

Two pieces of Kjeltab tablet and 15 ml of H_2SO_4 were added to each tube. The tubes were then placed on a Tecator 2020 Digester pre heated to 420°C. Digestion took a total minimum of 1 hr, ensuring that the resulting digestion was clear with no black particles present. The tubes were allowed to cool for 5 min in the digester, and 10 to 15 min after being removed. The samples were then distilled and titrated on the Kjeltec Auto System.

Fat (acid hydrolysis with diethyl ether). Lipid extraction cups containing two to four glass beads were placed in a drying oven at 105°C for a minimum of 30 min, cooled in a desiccator for 30 min, and the weight recorded. Glass filter thimbles were then placed inside glass tubes sealed by a rubber ring. One gram of celite was added to the thimbles. One to two grams (measured to 4 decimal places) of finely ground and homogenised sample (as above) was then added to the same thimble.

Pre extraction - In samples expected to contain more than 10% fat, a pre extraction stage was required. In this case, the thimbles were placed into the extraction unit (Soxtec System HT 1043 extraction unit with Soxtec System HT 1046 service unit, FOSS Analytical AB, Sweden).

Twenty millilitres of diethyl ether was added to each extraction cup, which were then placed in the extraction unit. Rinsing on the Soxtec unit was carried out for 15 min, followed by solution collection for 10 min, evaporation for 5 min in the unit and 1 to

3 min in a fume cupboard, following which the cups were placed in an incubator for 30 min at 105°C. They were then cooled for 30 min in a desiccator, and weighed.

Hydrolysis – One gram of celite was added to a series of protein tubes, and the pre extracted sample with celite was transferred to each tube in order. If pre extraction was not required, the sample was weighed into a protein tube directly with 1 g celite.

One hundred millilitres of 4 M HCl was added to each tube, and 1 g celite was added to new glass filter thimbles which were all then placed in the hydrolysis unit. The unit was started and the solution boiled carefully for 30 min, after which they were cooled. 100 ml of distilled water was added to each tube, and the lipid was absorbed onto the filter thimble under vacuum. Each tube was then washed out with warm distilled water which was also absorbed under vacuum (2 x 50 ml washes). Finally the protein tubes were cleaned with cotton wool and a little acetone which was then placed into the corresponding filter thimble. Thimbles were then placed in a microwave on defrost for 30 min.

Extraction - Clean extraction cups with 2 to 4 glass beads were placed in a drying oven for a minimum of 30 min and allowed to cool in a desiccator, and then weighed to 4 decimal places. The glass filter thimbles with the dried samples were then placed in the Soxtec instrument, and 40 ml of diethyl ether was dispensed into each extraction cup in the extraction unit. The process consisted of boiling for 20 min, 40 min of rinsing, 10 min of collection, evaporation for 5 min in the unit and 1 to 3 min in a fume cupboard, following which the cups were placed in an incubator for 30 min at 105°C. They were then cooled for 30 min in a desiccator, and weighed

Appendix 3

Gastrointestinal morphometrics and blood chemistry

Gastrointestinal morphometrics. Samples of stomach, mid intestine and distal intestine were removed from Bouin's fixative and dehydrated in a saturated solution of lithium carbonate in 70% ethanol prior to processing. A tissue processor was then used to prepare the tissue for histology, using methods based on those described by Vogan *et al.* (2001). Tissues were dehydrated through a graded series of alcohols (70, 80, 90 and three 100% alcohols, each for 60 min) and cleared in Histoclear (National Diagnostics, UK) for 4 hr. The specimens were then placed into a 50:50 mixture of Histoclear/ molten paraffin wax and maintained at 60°C for 2 hr, after which they were transferred into 2 changes of 100% molten paraffin wax at 2 hr intervals at 60°C before finally embedding in paraffin wax.

Embedded samples were placed in a vacuum oven for 2 hr at 60°C to ensure effective embedding prior to allowing the wax to set. Sections of each tissue were cut at 5 μm, from three regions within the blocks, placed on microscope slides and subsequently stained with Cole's haematoxylin and eosin. Following staining, slides were mounted with DPX. For each fish six slides were produced for qualitative and morphometric analysis (2 stomach, 2 mid intestine, 2 distal intestine) with five or more sections of tissue on each slide. A microscope with X10, X40 and X100 objectives was used to study morphological variations between the sea bass fed the experimental feeds.

Morphometric analysis was based on the findings of Baeverfjord and Krogdahl (1996). The authors classified subacute non-infectious enteritis in Atlantic salmon fed soybean meal by four morphological characteristics in the gastrointestinal tract:

1) shortening of villus height
2) widening of the villi with increased amounts of connective tissue
3) infiltration of inflammatory cells in the *lamina propria*
4) loss of supranuclear vacuolisation of absorptive cells in intestinal epithelium

Measurements were converted into proportions to allow comparisons between fish of different sizes. These proportions were as follows:

- distal intestine/mid intestine diameter (Fig. 14) as a proportion of fish weight (μm/g)
- distal intestine/mid intestine muscle thickness (Fig. 14) as a proportion of distal intestine/mid intestine diameter (μm/μm)
- distal intestine/mid intestine villus width (Fig. 16) as a proportion of distal intestine/mid intestine diameter (μm/μm)
- distal intestine/mid intestine villus length (Fig. 15) as a proportion of distal intestine/mid intestine diameter (μm/μm)
- distal intestine/mid intestine *lamina propria* diameter (Fig. 16) as a proportion of distal intestine/mid intestine villus diameter (μm/μm)
- distal intestine/mid intestine quantity of villi (Fig. 15) as a proportion of distal intestine/mid intestine diameter (villus/μm)

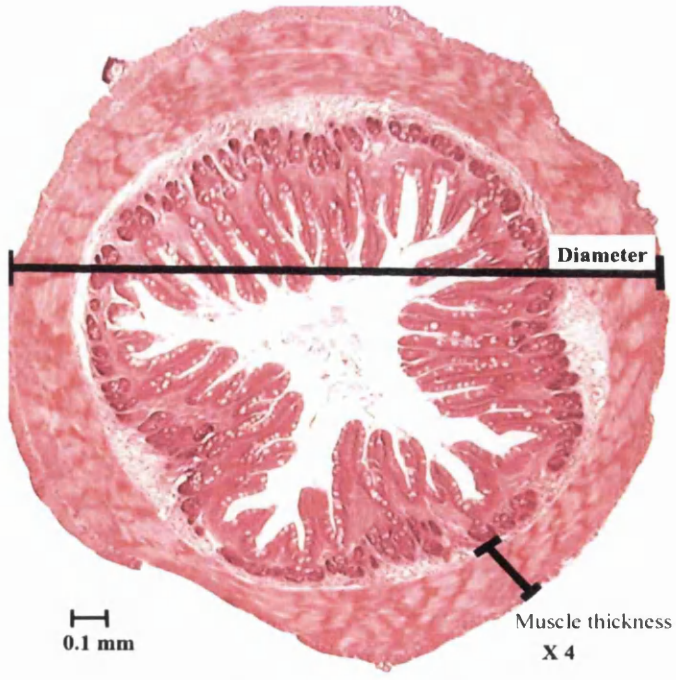

Figure 14: Transverse section through midgut of Atlantic cod (*Gadus morhua*) showing muscle area. Cole's haematoxylin and eosin stained. Diameter measured once for each section. Muscle thickness measured in 3 separate areas in each section. The measurement was not random; areas were chosen that best represented the true muscle thickness of the section. Both measurements were carried out at a x 4 objective (from Buckley (2006)).

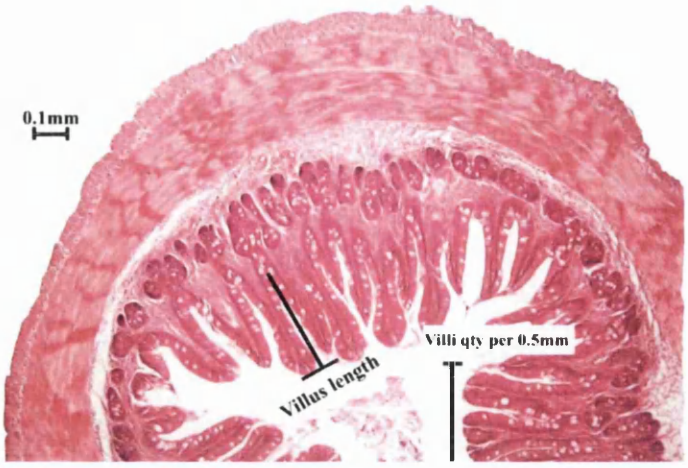

Figure 15: Transverse section through midgut of Atlantic cod (*Gadus morhua*) showing the methods used to measure villus length and density. The lengths of 5 villi were measured in each section, measured from the tip of the villus to the bottom of the fold as shown. A 0.5 mm line was used to assess the quantity of villi within a given area. If half of a villus encroached the line of measurement then that would also be included in the results as a 0.5 measurement. This was repeated 3 times for each section. Both measurements were carried out with a x 10 objective (from Buckley (2006)).

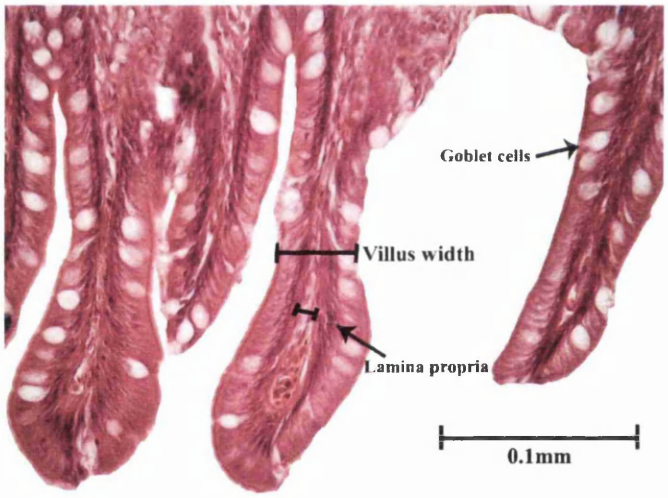

Figure 16: Transverse section of Atlantic cod (*Gadus morhua*) midgut showing the methods used to measure villus and *lamina propria* width. The widths of 5 villi were measured in each section, measured from the section of the villus that best represented the average width. The same place was used for all samples measured. Measurements of the *lamina propria* were taken from the same villi used for villus width. Five measurements were taken for each section. Both measurements were carried out with a x 40 objective (from Buckley (2006))

A further measurement was calculated which converted length and width measurements into a surface area value. An equation to calculate the surface area of a cylinder was used and manipulated for the purposes of the trial.

Cylinder surface area = $(2\ pi\ (r/2)^2)$ + $(2\ pi\ (r/2)\ hr)$

where R= radius (width of villus/2) and HR= length of villus.

To be accurate and best represent the structure of the villus, the equation had to be manipulated so that only one end was calculated i.e. villi only have one end exposed. Villi are not true cylinders as one end is connected to the gut and therefore not contributing to the surface area. The revised equation where only one of the cylinder tops is calculated therefore took the form:

Villus surface area = $(pi\ (r/2)^2) + (2\ pi\ (r/2)\ hr)$

During the qualitative analysis it was observed that the ends of villi were not flat as the equation implies. However the amount of measurements needed to fully calculate the bulbous end was not realistic in the allotted time scale. All ends are therefore assumed to be flat, and villi are all assumed to be symmetrical. Once villus surface area was calculated the surface area measurement was then converted into a proportion of gut diameter; this ensured that a comparison could be made between fish of different sizes.

Blood chemistry. Blood serum was analysed for total protein using the BCA colorimetric assay (Pierce-Warriner, U.K.). Reagents A and B were mixed together at a 50:1 ratio. Reagent A (68.6 ml) was added to reagent B (1.4 ml) to produce a total of 70 ml of reagent. A standard curve was produced according to the values in Table 1. Once the standards had been produced 50 µl of each was transferred to a 96 well plate. Reagent (200 µl) was then transferred to each standard sample. The standards were measured at 550 nm using a microplate reader after a 15 min incubation period.

Table 1: Concentrations required to obtain a standard curve for total protein assay.

BSA (µl)	3% NaCl (µl)	= Total protein (µg/ml)
50	0	2000
25	25	1000
20	30	800
15	35	600
10	40	400
5	45	200
2.5	47.5	100

In order to analyse blood serum for total protein, it was necessary to dilute it before the values were in range of the colorimetric assay. All serum samples were diluted (1:50) using 3% NaCl solution, and transferred to three wells of a 96 well plate giving three total protein measurements per fish. Reagent (200 µl) was transferred to each sample and left to incubate at 37°C for 15 min. Samples were measured at 550 nm. The absorbance values were multiplied by 50 to account for the dilution (1:50).

Aspartate aminotransferase (AST) activity was measured using a colorimetric assay (Randox Laboratories Ltd, UK). A standard curve was produced using the following procedure. Ten test tubes were prepared with the reagents as detailed in Table 2. 2, 4-Dinitrophenyl-hydrazine (1.0 ml) was then added to each of the 10 tubes. The 10 tubes were then mixed and incubated for 20 min at 25°C. Sodium hydroxide (10 ml) solution was then added to each tube to terminate the reaction. The resulting sample (200 µl) was then pipetted into the 96 well plate and read at 550 nm on a microplate reader. This was carried out in triplicate.

Table 2: Quantities of reagents needed for the production of an AST standard curve.

Tube number	Diluted pyruvate standard (ml)	Water (ml)	Buffer solution (ml)
1	0.00	0.20	1.00
2	0.05	0.20	0.95
3	0.10	0.20	0.90
4	0.15	0.20	0.85
5	0.20	0.20	0.80
6	0.25	0.20	0.75
7	0.30	0.20	0.70
8	0.35	0.20	0.65
9	0.40	0.20	0.60
10	0.45	0.20	0.55

Dilute serum (100 µl) and buffer (500 µl) were incubated at 25°C for 30 min. 2, 4-dinitrophenyl-hydrazine (500 µl) was then transferred to the mixture and allowed to incubate at 25°C for 20 min. Sodium hydroxide was then added to terminate the reaction. The sample (200 µl) was then pipetted into a 96 well plate and measured at 550 nm.

Alanine aminotransferase activity was measured using a colorimetric assay (Randox Laboratories Ltd, UK). A standard curve was produced from the following procedure. Test tubes were prepared with the reagent as detailed in Table 3. 2, 4-Dinitrophenyl-hydrazine (1.0 ml) was then added to each of the 10 tubes, mixed and incubated for 20 min at 25°C. Sodium hydroxide (10 ml) solution was then added to each tube to terminate the reaction. The resulting sample (200 µl) was then pipetted into the 96 well plate. This was carried out in triplicate.

Table 3: Quantities of reagents needed for the production of an Alanine aminotransferase standard curve

Tube number	Pyruvate standard (ml)	Water (ml)	Buffer solution (ml)
1	0.00	0.20	1.00
2	0.05	0.20	0.95
3	0.10	0.20	0.90
4	0.15	0.20	0.85
5	0.20	0.20	0.80
6	0.25	0.20	0.75
7	0.30	0.20	0.70
8	0.35	0.20	0.65
9	0.40	0.20	0.60
10	0.45	0.20	0.55

Dilute serum (100 µl) and buffer (500 µl) were incubated at 25°C for 30 min. 2, 4-dinitrophenyl-hydrazine (500 µl) was then transferred to the mixture and left to incubate at 25°C for 20 min. Sodium hydroxide was then added to the mixture to terminate the reaction. The sample (200 µl) was then pipetted into a 96 well plate and read at 550 nm on a microplate reader.

Appendix 4

Manuscript submitted to and presented at Aquaculture Europe 2008 - Resource Management; natural, human, and material resources for the sustainable development of aquaculture), Krakow, Poland.

CHARACTERISATION OF AQUACULTURE EFFLUENT: DOES PROTEIN SOURCE IN DIETS FOR MARINE FARMED FISH PLAY A ROLE IN DETERMINING THE PHYSICAL QUALITIES OF FISH FAECAL WASTE?

James, J. M.[a], Lupatsch, I.[a], Vikeså, V.[b], Morris, P.[c], Lovitt, R. W.[d], Shields, R. J.[a]

[a] Centre for Sustainable Aquaculture Research, Swansea University, Swansea, Wales, SA2 8PP;

[b] Skretting Aquaculture Research Centre, Sjøhagen 3, 4016 Stavanger, Norway;

[c] Skretting, Inverbreakie Industrial Estate, Invergordon, Ross-shire, Scotland, IV18 0QR

[d] Centre for Complex Fluid Processing, Multidisciplinary Nanotechnology Centre, Swansea University, Swansea, Wales, SA2 8PP

Introduction

Suspended solids in recirculating aquaculture systems (RAS), consisting mainly of faecal particles, are a significant part of the total nutrient load. Failure to effectively remove them from the culture water can seriously impair water quality and fish growth performance. Knowledge of the total amount of faeces produced and its physical properties in relation to feed composition is important for predicting total particulate loading and to optimise methods for suspended solids removal.

In this study, we have quantified the volume of faeces produced by marine fish receiving different diets and have characterised the physical properties of the faeces in terms of particle size distribution and zeta potential. Since the dimensions of fish faecal particles are known to range over nearly four orders of magnitude, from *circa* 1 μm to 2,000 μm (Bagnold and Barndorffnielsen, 1980), it is helpful to ascertain

specific size distributions for individual fish species and diets, in order to optimize RAS water treatment processes. In the current study, 'fine' particles have been defined as those less than 250 μm in diameter (i.e. passing through a 250 μm mesh), which are considered to have the greatest impact on suspended solids load due to slow rates of settling.

The arrangement of charge on the surface of a suspended particle (the dispersed phase) and the charges of counter-ions (oppositely charged) and co-ions (like charged) in the surrounding liquid (the continuous phase) is organised into an electrical double-layer around the particle. This double layer consists of the Stern (inner) layer where ions are tightly bound to the particle surface and the diffuse (outer) layer consisting of a loose cloud of ions (Hunter, 1988, Shaw, 1992). The distribution of charge can be quantified by measuring the zeta potential (ζ) of the suspended particles, which refers to the electrostatic potential at the surface of shear between the charged surface and the electrolyte solution (Shaw, 1992). Measurement of the zeta potential of fine faecal particles allows us to determine how these particles will behave relative to one another in suspension. This is because any charge, positive or negative, will lead to electrostatic repulsion between particles, reducing the likelihood of collision and flocculation. Reducing this net charge allows particles to get much closer to one another in the suspension, leading to the production of larger particles and flocs. The point at which the net charge reaches zero is defined at the isoelectric point, or IEP (Shaw, 1992), and is where the likelihood of collision and flocculation is at its highest.

The nature of how effluents flow and deform, in particular in relation to their viscosity, can have significant implications in effluent pumping and transport (Sanin, 2002). The viscosity of fish faecal wastes can influence the stability of the faecal material, in turn affecting particle size distributions and therefore effluent removal potential (Brinker *et al.*, 2005, Amirkolaie *et al.*, 2006). It is therefore desirable to achieve a balance between feed compositions that encourage a high faecal removal potential (high viscosity/large particle sizes), but which also encourage low cost and trouble free pumping and pipeline transport (low viscosities), without impairing feed digestibility.

Separate trials were conducted with juvenile Atlantic cod (*Gadus morhua*) and European sea bass (*Dicentrarchus labrax*) in a 60,000 L marine RAS at the Centre for Sustainable Aquaculture Research (CSAR), Swansea University. During the initial phase of each trial, replicated groups of fish were offered a series of experimental diets and regular measurements made of survival rate, growth rate and feed conversion ratio. Sea bass were grown from an average body weight of 47.30 g to 137.37 g in 97 days, while cod were grown from an average body weight of 80.23 g to 229.46 g in 112 days. Following this period of growth monitoring, faecal samples were collected from individual fish and pooled, in order to calculate feed digestibility based on yttrium oxide-labelled diets. Sea bass faeces were collected by repeated manual stripping of live fish, whereas cod faeces were collected on a single occasion, post-euthanasia. Both studies utilised experimental diets in which 25% of the fish meal component had been substituted by a plant protein, specifically high protein soya meal (HiPro soya), low protein soya meal (LoPro soya), soy protein concentrate (soy conc.), wheat gluten, and lupin seed meal for both cod and sea bass, plus dehulled and whole faba beans for sea bass only (see Table 1).

Physical analyses of cod and sea bass faeces were carried out at the Centre for Complex Fluid Processing, Swansea University. Particle sizing was carried out on a Malvern Mastersizer. Due to the way in which the data are reported from this instrument, particles less than 240 μm in size were considered as 'fine' in this instance. A Malvern Zetasizer was additionally used to determine zeta potential of the fine particle fraction from sea bass faeces, in a range of buffered pH solutions from pH 2 to pH 10 (insufficient sample volume was available for zeta potential analysis of cod faeces). Faecal samples were additionally subjected to rheological analysis, to assess their viscoelastic properties (data not shown).

Results

The formulations and proximate compositions for all diets used in these studies are shown in Table 1.

For both Atlantic cod (Fig. 1) and European sea bass (Fig. 2), faecal matter produced by fish receiving plant protein-substituted diets generally contained a lower proportion of fine particles in relation to total faecal production, based on feed digestibility calculations, compared to the fish meal control. The exception to this was a wheat gluten feed, which showed the highest proportion of fine particles in relation to total production of faeces for both species. The soy protein concentrate based feed yielded the lowest proportion of fine faecal particles in the case of cod, but in the case of sea bass this was found to be true for the whole faba bean feed. However, plant protein based diets generally resulted in a greater absolute mass of fine faecal particles due to lower feed digestibility, with the exception of soy protein concentrate and dehulled faba bean based diets for cod and sea bass respectively. In Atlantic cod, the highest absolute mass of fine faecal particles was produced by a feed containing wheat gluten, whereas a feed containing lupin produced the highest mass of fine particles in sea bass.

For Atlantic cod, the fish meal feed produced the least total mass of fine faecal particles, at 114 g dry matter kg^{-1} fish produced. Among the diets tested for cod, this would result in the least potential water quality and effluent removal issues in a RAS. All three soya-based diets and the wheat gluten-based feed yielded similar quantities of fine faecal particles, producing 44% more fine particles on average than the fish meal feed; soy protein concentrate performed best in this respect among the tested plant protein sources. For European sea bass, the feed containing dehulled faba beans produced the least mass of fine faecal particles, at 131 g of dry matter kg^{-1} fish produced. Fish meal, wheat gluten, and hull-on faba bean-based diets resulted in similar values, producing 31% more fine particles on average than dehulled faba beans. For both the Atlantic cod and European sea bass, the lupin-based feed yielded the highest mass of fine faecal particles, at 265 and 255 g dry matter kg^{-1} fish produced respectively.

Table 2 summarises results for each fish species on feed conversion ratio (FCR) of feed dry matter, and faecal production (both absolute and as fine particles) per kilogram of fish produced. For cod, the best FCR was recorded among those fish fed the fish meal feed (FCR 0.65), whereas the worst performing feed in this respect was that containing lupin (FCR 1.14). The FCR for sea bass was consistently higher than

for cod, with the feed containing soy protein concentrate performing best (FCR 1.13) and that containing lupin performing worst (FCR 1.22).

Zeta potential analyses for fine faecal material from both fish species across all diets revealed an isoelectric point between pH 2.34 and 2.85 and similar patterns of charge, with a rapid drop to between -5.8 and -11.7 mV at pH 4.0 to 4.5, followed by a small amount of fluctuation at higher pH values. At a pH of 8.0, as maintained in the marine RAS, the range of charge across all diets was -6.8 to -11.5 mV.

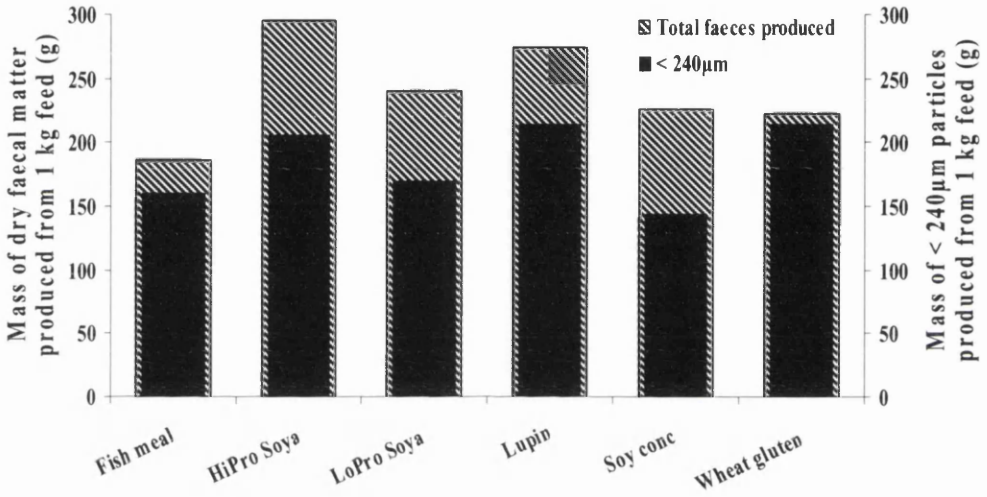

Figure 1: Mass of total faecal material and mass of faecal particles below 240 µm produced in relation to feed (g faeces kg^{-1} feed fed both expressed in dry matter) for Atlantic cod (*Gadus morhua*).

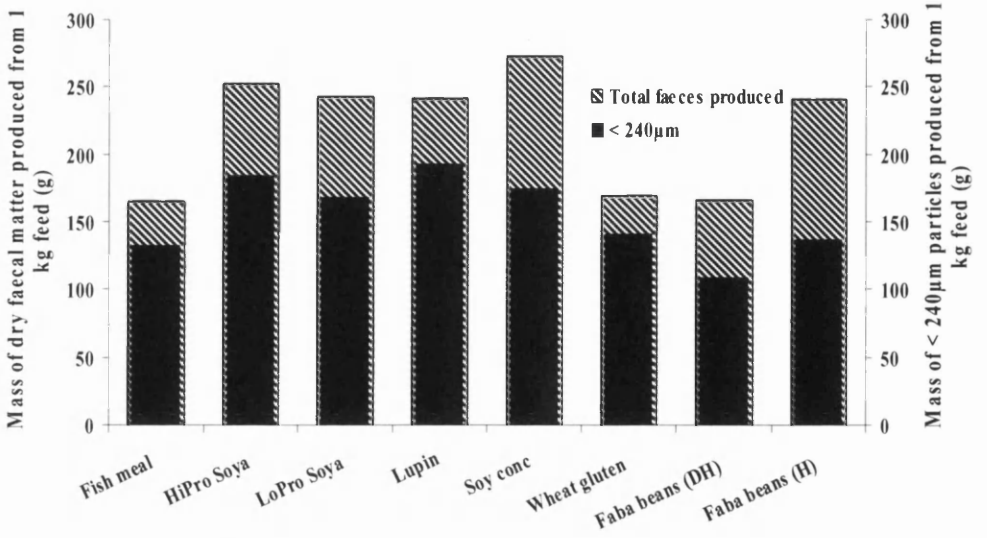

Figure 2: Mass of total faecal material and mass of faecal particles below 240 µm produced in relation to feed (g faeces kg^{-1} feed fed both expressed in dry matter) for European sea bass (*Dicentrarchus labrax*).

Table 1: Feed formulations and proximate composition of experimental diets fed to Atlantic cod (*Gadus morhua*) (first 6 diets) an (all diets)

Ingredients	**Fish meal Control**	**HiPro soya**	**LoPro soya**	**Soy conc.**	**Wheat Gluten**	**Lupin**	**Hull**
Fish meal	73.19	56.29	53.96	50.58	46.78	59.40	66.6
Fish oil	9.83	11.10	10.79	11.56	10.70	9.09	8.00
HiPro soya (48%)	-	25.00	-	-	-	-	-
LoPro soy (46%)	-	-	25.00	-	-	-	-
soy conc.	-	-	-	25.00	-	-	-
Wheat gluten	-	-	-	-	25.00	-	-
Lupin seed meal	-	-	-	-	-	25.00	-
Hull-on faba beans	-	-	-	-	-	-	25.0
Dehulled faba beans	-	-	-	-	-	-	-
Mineral and vitamin premix	0.29	0.29	0.29	0.29	0.29	0.29	0.29
Yttrium oxide premix	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Wheat starch	16.60	7.23	9.85	12.46	17.13	6.11	0.00
Proximate composition%							
Dry matter	92.48	92.49	91.92	91.54	91.74	92.38	92.8
Lipid	15.81	16.55	16.26	15.74	15.80	15.76	15.5
Protein	52.78	51.00	51.68	50.94	52.39	49.83	52.8
Ash	9.93	9.78	9.78	9.04	6.53	10.04	11.1

Table 2: Feed conversion ratio (FCR) as determined during growth trials and faeces and fine particle production as determined usi
shown as kg of waste per kg of fish produced, for both Atlantic cod (*Gadus morhua*) and European sea bass (*Dicentrarchus labrax*

	Cod			**Sea bass**	
Feed	**FCR, kg feed (DM) / kg growth**	**Faeces, kg (DM) / kg growth**	**Particles < 240 µm, kg (DM) / growth**	**FCR, kg feed (DM) / kg growth**	**Faece growt**
Fish meal	0.65	0.132	0.114	1.15	0.206
HiPro soya	0.74	0.235	0.164	1.17	0.320
LoPro soya	0.90	0.237	0.167	1.20	0.319
Lupin	1.14	0.338	0.265	1.22	0.319
Soy conc.	0.98	0.243	0.155	1.13	0.340
Wheat gluten	0.72	0.176	0.170	1.16	0.217
Faba beans (DH)	-	-	-	1.14	0.200
Faba beans (H)	-	-	-	1.14	0.296

Broad differences were observed between fish species in feed conversion ratios, irrespective of feed composition, such that Atlantic cod produced less faeces per kg of feed ingested than European sea bass. Overall, those diets in which fish meal had been substituted by plant proteins tended to produce a lower proportion of fine faecal particles compared to the fish meal control feed. However, when the total volume of faeces produced was taken into account via feed digestibility measurements, the 'substituted' diets generally yielded a greater absolute mass of fine faecal particles.

The above illustrates the importance of combining faecal particle size data with feed digestibility data when evaluating formulated diets in terms of their effects on nutrient loading. The results from these trials shows us that, in the case of Atlantic cod, the fish meal based feed would result in the least potential water quality and effluent removal issues in a RAS. In the case of European sea bass, this would be the dehulled faba bean based substituted feed. In both cases, using a feed containing lupin seed meal would cause the greatest potential negative effects. Such information could be applied in commercial RAS to select the most appropriate diets to minimise total particulate loading and to fine tune solids removal systems according to the particle size distributions of faeces

The zeta potential analysis of fine faecal particles from European sea bass suggests that there will be electrostatic repulsion under farm conditions (i.e. pH in the range 7.5 to 8.0), due to the moderately negative values obtained. However, the faecal suspension would remain within the 'unstable' zone of zeta potential (where flocculation can occur), between ± 30 mV. This inherent instability would tend to promote a level of flocculation of the fine particles, leading to a reduction in the fine particle loading. This instability could potentially be enhanced through the addition of a positively charged material to the diets which, if egested with its charge largely unchanged along with the faeces, would act to attract the particles showing a negative zeta potential and further improve the settling properties of the faecal solids. Further research is recommended to examine the effects of incorporating such charged particles or fibres into formulated diets.

Conclusion

The ability to incorporate information on several aspects of feed digestibility, faecal production and particulate waste properties enables detailed estimates of effluent impact to be made for fish reared in RAS. It can be seen that by simply determining the anticipated volume or mass of faeces through digestibility studies, information on what may happen once the waste is suspended in the recirculating water and how the effluent treatment system may deal with it in a RAS is lacking. By employing the methods shown here, it is possible to predict not only how much waste will be produced throughout the growth cycle of a given species on a given feed, but also what the physical makeup of that waste will be, a tool which could be applied to tailor the filtration and effluent treatment system to the fish species and feed type in question. Furthermore, the potential to alter the charge distribution on faecal particles may allow the proportion of fine particles to be reduced for any feed. This would not necessarily affect the absolute mass of waste that is produced, but may aid in the removal of that waste in RAS through larger particle sizes and more rapid settling.

These data show that different dietary protein sources have a noticeable and measurable effect upon the physical makeup of effluents from aquaculture operations, and that these effects may be enhanced by further engineering the diets. It is suggested that these differences and improvements could have major impacts upon the efficiency of solids removal and water quality in RAS. It is also probable that the particle size distributions of effluents will have an effect on open water culture systems, but in such cases it may be desirable to select for diets that produce a greater proportion of fine particles, to reduce potential benthic smothering effects. With the addition of rheological data, we hope that a complete picture of how protein source affects effluent characteristics will emerge, and this will enable feed manufacturers to better design diets with the aim of minimising effluent loads and enabling more efficient effluent removal processes.

Acknowledgements

This work was funded through the generous support of Skretting and Swansea University. For laboratory analyses and technical support we also extend our thanks

to the staff of the Skretting Aquaculture Research Centre in Norway, and the Centre for Sustainable Aquaculture Research, Swansea.

References

Amirkolaie, A.K., Verreth, J.A.J & Schrama, J.W. (2006) Effect of gelatinization degree and inclusion level of dietary starch on the characteristics of digesta and faeces in Nile tilapia (*Oreochromis niloticus* (L.)). *Aquaculture*, **260**, 194-205.

Bagnold, R.A. & Barndorffnielsen, O. (1980) The pattern of natural size distributions. *Sedimentology*, **27**, 199-207.

Brinker, A., Koppe, W. & Rosche, R. (2005) Optimised effluent treatment by stabilised trout faeces. *Aquaculture*, **249**, 125-144.

Sanin, F.D. (2002) Effect of solution physical chemistry on the rheological properties of activated sludge. *Water Sa*, **28** 207-211.

Shaw, D.J. (1992) *Introduction to Colloid and Surface Chemistry*, Butterworth Heinemann.

7 Glossary

Acidity constant - A quantitative measure of the strength of an acid in solution. It is the equilibrium constant for dissociation in the context of acid-base reactions.

Antinutritional factor – A substance which, when present in human or animal foods, reduces growth.

Apparent digestibility coefficient - percentage of the food ingested that is absorbed (incorporates protein digestibility and DM and organic matter digestibility).

Basicity constant - A quantitative measure of the strength of an alkali in solution. Related to the acidity constant by the simple relationship $pK_a + pK_b = 14$.

Biological oxygen demand - A measurement of the oxygen depletion by aerobic decomposition of organic matter by bacteria in a water sample incubated under controlled conditions over a period of time.

Electrophoretic mobility - Coefficient of proportionality between particle speed and electric field strength, in relation to hydrodynamic friction, which affects all bodies moving in viscous fluids.

Feed conversion ratio - A measure of an animal's efficiency in converting feed mass into increased body mass; the mass of the food eaten divided by the body mass gain, over a specified period of time.

Gastrointestinal evacuation time - Time taken for food items ingested to be passed through the GI tract during digestion.

Hepatosomatic index - Liver weight as a percentage of the whole body weight.

Integrated multi trophic aquaculture - A practice in which the by-products (wastes) from one species are recycled to become inputs (fertilisers, food) for another.

Isoelectric point - The pH at which a particular molecule or surface carries no net electrical charge.

Loss modulus (viscous modulus) - The mathematical description of a substance's tendency to be deformed viscously (i.e. permanently) when a force is applied to it (energy dissipation).

Rheology - The study of the flow and deformation of matter. Incorporates elastic and viscous moduli.

Rheological stability - The ability of a material of resist shear forces.

Storage modulus (elastic modulus) - The mathematical description of an object or substance's tendency to be deformed elastically (i.e. non-permanently) when a force is applied to it (energy storage).

Suspension stability - The likelihood of particles remaining in suspension, the stable state, in comparison to the likelihood of them flocculating and settling out of suspension, the unstable state (in relation to zeta potential).

Specific growth rate - The increase in mass per unit mass per unit time.

Zeta potential - Electrokinetic potential in colloidal systems, the potential difference between the dispersion medium and the stationary layer of fluid attached to the dispersed particle.

8 Bibliography

Adamidou, S., Nengas, I., Alexis, M., Foundoulaki, E., Nikolopoulou, D., Campbell, P., Karacostas, I., Rigos, G., Bell, G.J. & Jauncey, K. (1999) Apparent nutrient digestibility and gastrointestinal evacuation time in European seabass (*Dicentrarchus labrax*) fed diets containing different levels of legumes. *Aquaculture,* **289,** 106-112.

Adamidou, S., Nengas, I., Alexis, M., Foundoulaki, E., Nikolopoulou, D., Campbell, P., Karacostas, I., Rigos, G., Bell, G.J. & Jauncey, K. (2009) Apparent nutrient digestibility and gastrointestinal evacuation time in European seabass (*Dicentrarchus labrax*) fed diets containing different levels of legumes. *Aquaculture,* **289,** 106-112.

Adin, A., Soffer, Y. & Ben Aim, R. (1998) Effluent pretreatment by iron coagulation applying various dose pH combinations for optimum particle separation. *Water Science and Technology,* **38,** 27-34.

Aguedo, M., Wache, Y., Belin, J.M. & Teixeira, J.A. (2005) Surface properties of Yarrowia lipolytica and their relevance to gamma-decalactone formation from methyl ricinoleate. *Biotechnology Letters,* **27,** 417-422.

Ai, Q.H., Mai, K.S., Tan, B.P., Xu, W., Duan, Q.Y., Ma, H.M. & Zhang, L. (2006) Replacement of fish meal by meat and bone meal in diets for large yellow croaker, *Pseudosciaena crocea*. *Aquaculture,* **260,** 255-263.

Aksnes, A. & Opstvedt, J. (1998) Content of digestible energy in fish feed ingredients determined by the ingredient-substitution method. *Aquaculture,* **161,** 45-53.

Allan, G.L., Rowland, S.J., Parkinson, S., Stone, D.A.J. & Jantrarotai, W. (1999) Nutrient digestibility for juvenile silver perch *Bidyanus bidyanus*: development of methods. *Aquaculture,* **170,** 131-145.

Allan, G.L., Parkinson, S., Booth, M.A., Stone, D.A.J., Rowland, S.J., Frances, J. & Warner-Smith, R. (2000) Replacement of fish meal in diets for Australian silver perch, *Bidyanus bidyanus*: I. Digestibility of alternative ingredients. *Aquaculture,* **186,** 293-310.

Amaral, P.F.F., Lehocky, M., Barros-Timmons, A.M.V., Rocha-Leao, M.H.M., Coelho, M.A.Z. & Coutinho, J.A.P. (2006) Cell surface characterization of Yarrowia lipolytica IMUFRJ 50682. *Yeast,* **23,** 867-877.

Amirkolaie, A.K., Leenhouwers, J.I., Verreth, J.A.J. & Schrama, J.W. (2005) Type of dietary fibre (soluble versus insoluble) influences digestion, faeces characteristics and faecal waste production in Nile tilapia (Oreochromis niloticus L.). *Aquaculture Research,* **36,** 1157-1166.

Amirkolaie, A.K., Verreth, J.A.J. & Schrama, J.W. (2006) Effect of gelatinization degree and inclusion level of dietary starch on the characteristics of digesta and faeces in Nile tilapia (*Oreochromis niloticus* (L.)). *Aquaculture,* **260,** 194-205.

Andersen, F.A. (2005) Amended final report on the safety assessment of polyacrylamide and acrylamide residues in cosmetics. *International Journal of Toxicology,* **24,** 21-50.

Anon (2001) Sustainability of fish meal and oil supply. *Paper presented at Scottish-Norwegian Conference on Sustainable Futures for Marine Fish Farming,* www.iffo.org.uk/tech/sterling.htm.

Aslaksen, M.A., Kraugerud, O.F., Penn, M., Svihus, B., Denstadli, V., Jorgensen, H.Y., Hillestad, M., Krogdahl, A. & Storebakken, T. (2007) Screening of nutrient digestibilities and intestinal pathologies in Atlantic salmon, *Salmo salar*, fed diets with legumes, oilseeds, or cereals. *Aquaculture,* **272,** 541-555.

Atkins, P.W. & Jones, L.L. (2002) *Chemical Principles - The Quest For Insight (2d ed),* W. H. Freeman and Company, New York.

Austreng, E., Storebakken, T., Thomassen, M.S., Refstie, S. & Thomassen, Y. (2000) Evaluation of selected trivalent metal oxides as inert markers used to estimate apparent digestibility in salmonids. *Aquaculture,* **188,** 65-78.

Azaza, M.S., Wassim, K., Mensi, F., Abdelmouleh, A., Brini, B. & Kraiem, M.M. (2009) Evaluation of faba beans (*Vicia faba* L. var. *minuta*) as a replacement for

soybean meal in practical diets of juvenile Nile tilapia *Oreochromis niloticus*. *Aquaculture,* **287,** 174-179.

Baeverfjord, G. & Krogdahl, A. (1996) Development and regression of soybean meal induced enteritis in Atlantic salmon, *Salmo salar* L, distal intestine: A comparison with the intestines of fasted fish. *Journal of Fish Diseases,* **19,** 375-387.

Bagnold, R.A. & Barndorffnielsen, O. (1980) The Pattern of Natural Size Distributions. *Sedimentology,* **27,** 199-207.

Barrientos, M. (2009) Commodity Prices. *Index Mundi,* www.indexmundi.com/commodities/.

Berge, G.M., Grisdale-Helland, B. & Helland, S.J. (1999) Soy protein concentrate in diets for Atlantic halibut (*Hippoglossus hippoglossus*). *Aquaculture,* **178,** 139-148.

Bjornsson, B. & Olafsdottir, S.R. (2006) Effects of water quality and stocking density on growth performance of juvenile cod (*Gadus morhua* L.). *Ices Journal Of Marine Science,* **63,** 326-334.

Bonaldo, A., Roem, A.J., Pecchini, A., Grilli, E. & Gatta, P.P. (2006) Influence of dietary soybean meal levels on growth, feed utilization and gut histology of Egyptian sole (*Solea aegyptiaca*) juveniles. *Aquaculture,* **261,** 580-586.

Bonaldo, A., Roem, A.J., Fagioli, P., Pecchini, A., Cipollini, I. & Gatta, P.P. (2008) Influence of dietary levels of soybean meal on the performance and gut histology of gilthead sea bream (*Sparus aurata* L.) and European sea bass (*Dicentrarchus labrax* L.). *Aquaculture Research,* **39,** 970-978.

Boonyaratpalin, M., Suraneiranat, P. & Tunpibal, T. (1998) Replacement of fish meal with various types of soybean products in diets for the Asian seabass, *Lates calcarifer*. *Aquaculture,* **161,** 67-78.

Booth, M.A., Allan, G.L., Frances, J. & Parkinson, S. (2001) Replacement of fish meal in diets for Australian silver perch, *Bidyanus bidyanus* IV. Effects of dehulling and protein concentration on digestibility of grain legumes. *Aquaculture,* **196,** 67-85.

Borges, M.T., Morais, A. & Castro, P.M.L. (2003) Performance of outdoor seawater treatment systems for recirculation in an intensive turbot (*Scophthalmus maximus*) farm. *Aquaculture International,* **11,** 557-570.

Boyd, C.E. & Queiroz, J.F. (2001) Nitrogen, phosphorus loads vary by system: USEPA should consider system variables in setting new effluent rules In *The Advocate (December)*, pp. 84 - 86.

Brambilla, F., Antonini, M., Ceccuzzi, P., Terova, G. & Saroglia, M. (2008) Foam fractionation efficiency in particulate matter and heterotrophic bacteria removal from a recirculating seabass (*Dicentrarchus labrax*) system. *Aquacultural Engineering,* **39,** 37-42.

Brattelid, T. & Smith, A.J. (2000) Guidelines for reporting the results of experiments on fish. *Laboratory Animals,* **34,** 131-135.

Brinker, A., Schroder, H.G. & Rosch, R. (2005a) A high-resolution technique to size suspended solids in flow-through fish farms. *Aquacultural Engineering,* **32,** 325-341.

Brinker, A., Koppe, W. & Rosch, R. (2005b) Optimised effluent treatment by stabilised trout faeces. *Aquaculture,* **249,** 125-144.

Brinker, A. & Rosch, R. (2005c) Factors determining the size of suspended solids in a flow-through fish farm. *Aquacultural Engineering,* **33,** 1-19.

Brinker, A., Koppe, W. & Rosch, R. (2005d) Optimizing trout farm effluent treatment by stabilizing trout feces: A field trial. *North American Journal of Aquaculture,* **67,** 244-258.

Brinker, A. (2007) Guar gum in rainbow trout (*Oncorhynchus mykiss*) feed: The influence of quality and dose on stabilisation of faecal solids. *Aquaculture,* **267,** 315-327.

Brinker, A. (2008) Improving the mechanical characteristics of faecal waste in rainbow trout: the influence of fish size and treatment with a non-starch polysaccharide (guar gum). *Aquaculture Nutrition,* ***In press.***

Buckley, J. (2006) Intestinal morphometrics and blood chemistry of cod (*Gadus morhua*) fed plant protein-supplemented diets. MSc dissertation, Swansea University.

Burel, C., Boujard, T., Corraze, G., Kaushik, S.J., Boeuf, G., Mol, K.A., Van der Geyten, S. & Kuhn, E.R. (1998) Incorporation of high levels of extruded lupin in diets for rainbow trout (*Oncorhynchus mykiss*): nutritional value and effect on thyroid status. *Aquaculture,* **163,** 325-345.

Burel, C., Boujard, T., Kaushik, S.J., Boeuf, G., Van der Geyten, S., Mol, K.A., Kuhn, E.R., Quinsac, A., Krouti, M. & Ribaillier, D. (2000a) Potential of plant-protein sources as fish meal substitutes in diets for turbot (*Psetta maxima*): growth, nutrient utilisation and thyroid status. *Aquaculture,* **188,** 363-382.

Burel, C., Boujard, T., Tulli, F. & Kaushik, S.J. (2000b) Digestibility of extruded peas, extruded lupin, and rapeseed meal in rainbow trout (*Oncorhynchus mykiss*) and turbot (*Psetta maxima*). *Aquaculture,* **188,** 285-298.

Bury, N.R., Grosell, M., Wood, C.M., Hogstrand, C., Wilson, R.W., Rankin, J.C., Busk, M., Lecklin, T. & Jensen, F.B. (2001) Intestinal iron uptake in the European flounder (*Platichthys flesus*). *Journal of Experimental Biology,* **204,** 3779-3787.

Bury, N.R., Walker, P.A. & Glover, C.N. (2003) Nutritive metal uptake in teleost fish. *Journal of Experimental Biology,* **206,** 11-23.

Cai, K.Y., Frant, M., Bossert, J., Hildebrand, G., Liefeith, K. & Jandt, K.D. (2006) Surface functionalized titanium thin films: Zeta-potential, protein adsorption and cell proliferation. *Colloids and Surfaces B-Biointerfaces,* **50,** 1-8.

Carriquiriborde, P., Handy, R.D. & Davies, S.J. (2004) Physiological modulation of iron metabolism in rainbow trout (*Oncorhynchus mykiss*) fed low and high iron diets. *Journal of Experimental Biology,* **207,** 75-86.

Carter, C.G., Lewis, T.E. & Nichols, P.D. (2003) Comparison of cholestane and yttrium oxide as digestibility markers for lipid components in Atlantic salmon (*Salmo salar* L.) diets. *Aquaculture,* **225,** 341-351.

Castleman, L.S. (2007) Studies on the effects of plant protein substitution on digestive tract histology and blood parameters of European sea bass (*Dicentrarchus labrax*). MSc dissertation, Swansea University.

Chen, B.H., Lee, S.J. & Lee, D.J. (2005) Rheological characteristics of the cationic polyelectrolyte flocculated wastewater sludge. *Water Research,* **39,** 4429-4435.

Chen, S., Coffin, D.E. & Malone, R.F. (1993) Production, characteristics, and modelling of aquaculture sludge from a recirculating aquaculture system using granular media biofilter In *Techniques for Modern Aquaculture* (Wang, J.K. ed.), pp. 16 - 25. American Society of Aquacultural Engineers, St. Joseph, MI.

Chen, S., Stechey, D. & Malone, R.F. (1994) Suspended solids control in recirculating aquaculture systems. In *Development in Aquaculture and Fisheries Science 27, Aquaculture Water Reuse Systems: Engineering Design and Management.* (Timmons, M.B. & Losordo, T.M. eds.), pp. 61-100. Elsevier, Amsterdam.

Chen, S.L., Timmons, M.B., Bisogni, J.J. & Aneshansley, D.J. (1993a) Suspended solids removal by foam fractionation. *Progressive Fish-Culturist,* **55,** 69-75.

Chen, S.L., Coffin, D.E. & Malone, R.F. (1997) Sludge production and management for recirculating aquacultural systems. *Journal of the World Aquaculture Society,* **28,** 303-315.

Chen, Y.S., Beveridge, M.C.M. & Telfer, T.C. (1999) Settling rate characteristics and nutrient content of the faeces of Atlantic salmon, *Salmo salar* L., and the implications for modelling of solid waste dispersion. *Aquaculture Research,* **30,** 395-398.

Chen, Y.S., Beveridge, M.C.M., Telfer, T.C. & Roy, W.J. (2003) Nutrient leaching and settling rate characteristics of the faeces of Atlantic salmon (*Salmo salar* L.) and the implications for modelling of solid waste dispersion. *Journal Of Applied Ichthyology,* **19,** 114-117.

Chi, Z.M., Wang, F., Chi, Z., Yue, L.X., Liu, G.L. & Zhang, T. (2009) Bioproducts from *Aureobasidium pullulans*, a biotechnologically important yeast. *Applied Microbiology and Biotechnology,* **82,** 793-804.

Choct, M., Hughes, R.J., Trimble, R.P., Angkanaporn, K. & Annison, G. (1995) Nonstarch polysaccharide degrading enzymes increase the performance of broiler chickens fed wheat of low apparent metabolizable energy. *Journal of Nutrition,* **125,** 485-492.

Choi, S.M., Wang, X., Park, G.J., Lim, S.R., Kim, K.W., Bai, S.C. & Shin, I.S. (2004) Dietary dehulled soybean meal as a replacement for fish meal in fingerling and growing olive flounder *Paralichthys olivaceus* (Temminck et Schlegel). *Aquaculture Research,* **35,** 410-418.

Craig, S.R. & McLean, E. (2005) The organic aquaculture movement: a role for NuPro as an alternative protein source In *Nutritional Biotechnology in the Food and Feed Industry* (Jacques, K. & Lyons, P. eds.). Nottingham University Press, Nottingham.

Cripps, S.J. & Kelly, L.A. (1996) Reductions in waste from aquaculture In *Aquaculture and Water Resource Management* (Baird, D.J., *et al.* eds.), pp. 166 - 201. Blackwell Science, Oxford.

Cripps, S.J. & Bergheim, A. (2000) Solids management and removal for intensive land-based aquaculture production systems. *Aquacultural Engineering,* **22,** 33-56.

Cromey, C.J., Nickell, T.D. & Black, K.D. (2002) DEPOMOD - modelling the deposition and biological effects of waste solids from marine cage farms. *Aquaculture,* **214,** 211-239.

Cromey, C.J., Nickell, T.D., Treasurer, J., Black, K.D. & Inall, M. (2009) Modelling the impact of cod (*Gadus morhua* L.) farming in the marine environment—CODMOD. *Aquaculture,* **289,** 42-53.

Cromwell, G.L. (2006) *Rendered products in swine nutrition. In: Essential Rendering,* National Renderers Assoc, Alexandria, VA. .

D'Souza, N., Skonberg, D.I., Stone, D.A.J. & Brown, P.B. (2006) Effect of soybean meal-based diets on the product quality of rainbow trout fillets. *Journal of Food Science,* **71,** S337-S342.

Day, O.J. & Gonzalez, H.G.P. (2000) Soybean protein concentrate as a protein source for turbot *Scophthalmus maximus* L. *Aquaculture Nutrition,* **6,** 221-228.

De Arruda, L.F., Borghesi, R. & Oetterer, M. (2007) Use of fish waste as silage - A review. *Brazilian Archives of Biology and Technology,* **50,** 879-886.

De Schryver, P., Crab, R., Defoirdt, T., Boon, N. & Verstraete, W. (2008) The basics of bio-flocs technology: The added value for aquaculture. *Aquaculture,* **277,** 125-137.

DEFRA (2006) Project Report FC0934: Appraisal of the opportunity for offshore aquaculture in UK waters, pp. 48 - 55.

di Pietro, B. (2009) Plant potential In *Fish Farming International*, Vol. 36: 4, pp. 2. IntraFish, London.

Dias, J., Huelvan, C., Dinis, M.T. & Metailler, R. (1998) Influence of dietary bulk agents (silica, cellulose and a natural zeolite) on protein digestibility, growth, feed intake and feed transit time in European seabass (*Dicentrarchus labrax*) juveniles. *Aquatic Living Resources,* **11,** 219-226.

Dossantos, J. & Jobling, M. (1991) Factors affecting gastric evacuation in cod, *Gadus morhua* L, fed single meals of natural prey. *Journal of Fish Biology,* **38,** 697-713.

Drew, M.D., Borgeson, T.L. & Thiessen, D.L. (2007) A review of processing of feed ingredients to enhance diet digestibility in finfish. *Animal Feed Science and Technology,* **138,** 118-136.

Droppo, I.G., Krishnappan, B.G., Rao, S.S. & Ongley, E.D. (1995) Investigation of a sequential filtration technique for particle fractionation. *Environmental Science & Technology,* **29,** 546-550.

Dudley, R.W., Panchang, V.G. & Newell, C.R. (2000) Application of a comprehensive modeling strategy for the management of net-pen aquaculture waste transport. *Aquaculture,* **187,** 319-349.

Elberizon, I.R. & Kelly, L.A. (1998) Empirical measurements of parameters critical to modelling benthic impacts of freshwater salmonid cage aquaculture. *Aquaculture Research,* **29,** 669-677.

Engin, K. & Carter, C.G. (2002) Ingredient apparent digestibility coefficients for the Australian short-finned eel (*Anguilla australis australis*, Richardson). *Animal Science,* **75,** 401-413.

Escaffre, A.M., Kaushik, S. & Mambrini, M. (2007) Morphometric evaluation of changes in the digestive tract of rainbow trout (*Oncorhynchus mykiss*) due to fish meal replacement with soy protein concentrate. *Aquaculture,* **273,** 127-138.

Fagbenro, O. & Jauncey, K. (1995) Water stability, nutrient leaching and nutritional properties of moist fermented fish silage diets. *Aquacultural Engineering,* **14,** 143-153.

FAO (2009) Monthly Price and Policy Update, No. 7 July 2009. FAO.

Fernandes, T. & Tett, P. (2001) Marine Biology, Sediment Analysis - Graphical presentation and statistics. www.lifesciences.napier.ac.uk/teaching/MB/phidiag01.

Fernandes, T.F., Eleftheriou, A., Ackefors, H., Eleftheriou, M., Ervik, A., Sanchez-Mata, A., Scanlon, T., White, P., Cochrane, S., Pearson, T.H. & Read, P.A. (2001) The scientific principles underlying the monitoring of the environmental impacts of aquaculture. *Journal of Applied Ichthyology,* **17,** 181-193.

Fernandez, F., Miquel, A.G., Cumplido, L.R., Guinea, J. & Ros, E. (1996) Comparisons of faecal collection methods for digestibility determinations in gilthead sea bream. *Journal Of Fish Biology,* **49,** 735-738.

Fernandez, F., Miquel, A.G., Guinea, J. & Martinez, R. (1998) Digestion and digestibility in gilthead sea bream (*Sparus aurata*): The effect of diet composition and ration size. *Aquaculture,* **166,** 67-84.

Fjelldal, P.G., van der Meeren, T., Jorstad, K.E. & Hansen, T.J. (2009) A radiological study on vertebral deformities in cultured and wild Atlantic cod (*Gadus morhua*, L.) *Aquaculture,* **289,** 6-12.

Forde-Skjaervik, O., Refstie, S., Aslaksen, M.A. & Skrede, A. (2006) Digestibility of diets containing different soybean meals in Atlantic cod (*Gadus morhua*); comparison of collection methods and mapping of digestibility in different sections of the gastrointestinal tract. *Aquaculture,* **261,** 241-258.

Fournie, J.W., Wolfe, M.J., Wolf, J.C., Courtney, L.A., Johnson, R.D. & Hawkins, W.E. (2005) Diagnostic criteria for proliferative thyroid lesions in bony fishes. *Toxicologic Pathology,* **33,** 540-551.

Francis, G., Makkar, H.P.S. & Becker, K. (2001) Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. *Aquaculture,* **199,** 197-227.

Friedman, M., Brandon, D.L., Bates, A.H. & Hymowitz, T. (1991) Comparison Of A Commercial Soybean Cultivar And An Isoline Lacking The Kunitz Trypsin-Inhibitor - Composition, Nutritional-Value, And Effects Of Heating. *Journal Of Agricultural And Food Chemistry,* **39,** 327-335.

Friedman, M. & Brandon, D.L. (2001) Nutritional and health benefits of soy proteins. *Journal Of Agricultural And Food Chemistry,* **49,** 1069-1086.

Gaber, M.M. (2006) Partial and complete replacement of fish meal by broad bean meal in feeds for Nile tilapia, *Oreochromis niloticus*, L., fry. *Aquaculture Research,* **37,** 986-993.

Garcia-Ruiz, R. & Hall, G.H. (1996) Phosphorus fractionation and mobility in the food and faeces of hatchery reared rainbow trout (*Onchorhynchus mykiss*). *Aquaculture,* **145,** 183-193.

Glencross, B., Curnow, J., Hawkins, W. & Felsing, M. (2002) Evaluation of yellow lupin *Lupinus luteus* meal as an alternative protein resource in diets for sea-cage reared rainbow trout *Oncorhynchus mykiss. Journal Of The World Aquaculture Society,* **33,** 287-296.

Glencross, B. & Hawkins, W. (2004) A comparison of the digestibility of lupin (*Lupinus* sp.) kernel meals as dietary protein resources when fed to either, rainbow trout, *Oncorhynchus mykiss* or red seabream, *Pagrus auratus*. *Aquaculture Nutrition,* **10,** 65-73.

Glencross, B., Evans, D., Dods, K., McCafferty, P., Hawkins, W., Maas, R. & Sipsas, S. (2005) Evaluation of the digestible value of lupin and soybean protein concentrates and isolates when fed to rainbow trout, *Oncorhynchus mykiss*, using either stripping or settlement faecal collection methods. *Aquaculture,* **245,** 211-220.

Glencross, B., Hawkins, W., Evans, D., Rutherford, N., Dods, K., McCafferty, P. & Sipsas, S. (2008) Evaluation of the influence of *Lupinus angustifolius* kernel meal on dietary nutrient and energy utilization efficiency by rainbow trout (*Oncorhynchus mykiss*). *Aquaculture Nutrition,* **14,** 129-138.

Glencross, B.D., Carter, C.G., Duijster, N., Evans, D.R., Dods, K., McCafferty, P., Hawkins, W.E., Maas, R. & Sipsas, S. (2004) A comparison of the digestibility of a range of lupin and soybean protein products when fed to either Atlantic salmon (*Salmo salar*) or rainbow trout (*Oncorhynchus mykiss*). *Aquaculture,* **237,** 333-346.

Gowen, R.J. & Bradbury, N.B. (1987) The ecological impact of salmonid farming in coastal waters - A review. *Oceanography and Marine Biology,* **25,** 563-575.

Grisdale-Helland, B., Helland, S.J., Baeverfjord, G. & Berge, G.M. (2002) Full-fat soybean meal in diets for Atlantic halibut: growth, metabolism and intestinal histology. *Aquaculture Nutrition,* **8,** 265-270.

Hamre, K. (2006) Nutrition in cod (*Gadus morhua*) larvae and juveniles. *Ices Journal of Marine Science,* **63,** 267-274.

Hansen, A.C., Rosenlund, G., Karlsen, R., Olsvik, P.A. & Hemre, G.I. (2006) The inclusion of plant protein in cod diets, its effects on macronutrient digestibility, gut and liver histology and heat shock protein transcription. *Aquaculture Research,* **37,** 773-784.

Hansen, A.C., Karlsen, O., Rosenlund, G., Rimbach, M. & Hemre, G.I. (2007) Dietary plant protein utilization in Atlantic cod, *Gadus morhua* L. *Aquaculture Nutrition,* **13,** 200-215.

Hardy, R.W. (2000) Advances in the development of low-pollution feeds for salmonids In *The Advocate (April)*, pp. 63 - 74.

Hardy, R.W., Sealey, W.M. & Gatlin, D.M. (2005) Fisheries by-catch and by-product meals as protein sources for rainbow trout *Oncorhynchus mykiss. Journal of the World Aquaculture Society,* **36,** 393-400.

Hargrave, B.T. (2003) A Scientific Review of the Potential Environmental Effects of Aquaculture in Aquatic Ecosystems. Volume I: Far-field Environmental Effects of Marine Finfish Aquaculture. *Canadian Technical Report of Fisheries and Aquatic Sciences 2450*, 11 - 49.

Harif, T. & Adin, A. (2007) Characteristics of aggregates formed by electroflocculation of a colloidal suspension. *Water Research,* **41,** 2951-2961.

Hemre, G.I., Karlsen, O., Mangor-Jensen, A. & Rosenlund, G. (2003) Digestibility of dry matter, protein, starch and lipid by cod, *Gadus morhua*: comparison of sampling methods. *Aquaculture,* **225,** 225-232.

Hertrampf, J.W. & Piedad-Pascual, F. (2003) *Handbook on Ingredients for Aquaculture Feeds,* Kluwer Academic Publishers.

Hjorth, M., Christensen, M.L. & Christensen, P.V. (2008) Flocculation, coagulation, and precipitation of manure affecting three separation techniques. *Bioresource Technology,* **99,** 8598-8604.

Holby, O. & Hall, P.O.J. (1991) Chemical fluxes and mass balances in a marine fish cage farm. 2 Phosphorus. *Marine Ecology-Progress Series,* **70,** 263-272.

Holmer, M., Marba, N., Diaz-Almela, E., Duarte, C.M., Tsapakis, M. & Danovaro, R. (2007) Sedimentation of organic matter from fish farms in oligotrophic Mediterranean assessed through bulk and stable isotope (delta C-13 and delta N-15) analyses. *Aquaculture,* **262,** 268-280.

Hu, M.H., Wang, Y.J., Luo, Z., Zhao, M., Xiong, B.X., Qian, X.Q. & Zhao, Y.J. (2008) Evaluation of rendered animal protein ingredients for replacement of fish meal in practical diets for gibel carp, *Carassius auratus gibelio* (Bloch). *Aquaculture Research,* **39,** 1475-1482.

Hunter, R.J. (1988) *Zeta Potential In Colloid Science: Principles and Applications,* Academic Press.

Hunter, R.J. (1993) *Introduction to Modern Colloid Science,* Oxford University Press.

Imsland, A.K., Foss, A., Koedijk, R., Folkvord, A., Stefansson, S.O. & Jonassen, T.M. (2006) Short- and long-term differences in growth, feed conversion efficiency and deformities in juvenile Atlantic cod (*Gadus morhua*) startfed on rotifers or zooplankton. *Aquaculture Research,* **37,** 1015-1027.

Jean, K., Renan, M., Famelart, M.H. & Guyomarc'h, F. (2006) Structure and surface properties of the serum heat-induced protein aggregates isolated from heated skim milk. *International Dairy Journal,* **16,** 303-315.

Johnson, W. & Chen, S.L. (2006) Performance evaluation of radial/vertical flow clarification applied to recirculating aquaculture systems. *Aquacultural Engineering,* **34,** 47-55.

Kara, F., Gurakan, G.C. & Sanin, F.D. (2008) Monovalent cations and their influence on activated sludge floc chemistry, structure, and physical characteristics. *Biotechnology and Bioengineering,* **100,** 231-239.

Kelly, L.A., Bergheim, A. & Stellwagen, J. (1997) Particle size distribution of wastes from freshwater fish farms. *Aquaculture International,* **5,** 65-78.

Kikuchi, K. (1998) Use of defatted soybean meal as a substitute for fish meal in diets of Japanese flounder (*Paralichthys olivaceus*) In *8th International Symposium on Nutrition and Feeding of Fish*, pp. 3-11 Las Palmas, Spain.

Kim, H.S., Joo, D.J. & Lee, J. (1999) Flocculating properties of polycondensate of 1-butyl amine and epichlorohydrin. *Environmental Technology,* **20,** 117-120.

Kissil, G.W. & Lupatsch, I. (2004) Successful replacement of fishmeal by plant proteins in diets for the gilthead seabream, *Sparus aurata* L. *Israeli Journal Of Aquaculture-Bamidgeh,* **56,** 188-199.

Kolstad, K., Thorland, I., Refstie, T. & Gjerde, B. (2006) Genetic variation and genotype by location interaction in body weight, spinal deformity and sexual maturity in Atlantic cod (*Gadus morhua*) reared at different locations off Norway. *Aquaculture,* **259,** 66-73.

Kristensen, L. & Purslow, P.P. (2001) The effect of processing temperature and addition of mono- and di-valent salts on the heme- nonheme-iron ratio in meat. *Food Chemistry,* **73,** 433-439.

Kristiansen, R. & Cripps, S.J. (1996) Treatment of fish farm wastewater using sand filtration. *Journal of Environmental Quality,* **25,** 545-551.

Krogdahl, A., Bakke-McKellep, A.M. & Baeverfjord, G. (2003) Effects of graded levels of standard soybean meal on intestinal structure, mucosal enzyme activities, and pancreatic response in Atlantic salmon (*Salmo salar* L.). *Aquaculture Nutrition,* **9,** 361-371.

Langar, H. (1994) Relationship between dietary protein quality, feeding pattern and growth in seabass (*Dicentrarchus labrax*). *CIHEAM - Options Mediterraneennes,* 265-274.

Langer, J., Efthimiou, S., Rosenthal, H. & Bronzi, P. (1996) Drum filter performance in a recirculating eel culture unit. *Journal of Applied Ichthyology-Zeitschrift Fur Angewandte Ichthyologie,* **12,** 61-65.

Lee, P.G. & Lawrence, A.L. (2001) Feed Management for Recirculating Aquaculture Systems: Part I. Nutrition and Filtration Aspects In *The Advocate (February)*, pp. 27 - 28.

Leenhouwers, J.I., Santos, G.A., Schrama, J.W. & Verreth, J.A.J. (2004) Digesta viscosity and nutrient digestibility in European sea bass (*Dicentrarchus labrax*) in a fresh water and marine environment. *Aquaculture Europe - Extended Abstracts and Short Communications,* **34,** 475 - 476.

Leenhouwers, J.I., Adjei-Boateng, D., Verreth, J.A.J. & Schrama, J.W. (2006) Digesta viscosity, nutrient digestibility and organ weights in African catfish (*Clarias gariepinus*) fed diets supplemented with different levels of a soluble non-starch polysaccharide. *Aquaculture Nutrition,* **12,** 111-116.

Lemarie, G., Martin, J.L.M., Dutto, G. & Garidou, C. (1998) Nitrogenous and phosphorous waste production in a flow-through land-based farm of European seabass (*Dicentrarchus labrax*). *Aquatic Living Resources,* **11,** 247-254.

Li, C.B., Hein, S. & Wang, K. (2008b) Biosorption of chitin and chitosan. *Materials Science and Technology,* **24,** 1088-1099.

Li, X.Y., Zhai, X.D., Chu, H.P. & Zhang, J.J. (2008a) Characterization of the flocculation process from the evolution of particle size distributions. *Journal of Environmental Engineering-Asce,* **134,** 369-375.

Liu, F., Ai, Q.H., Mai, K.S., Tan, B.P., Ma, H.M., Xu, W., Zhang, W.B. & Liufu, Z.G. (2008) Effects of dietary binders on survival and growth performance of postlarval tongue sole, *Cynoglossus semilaevis* (Gunther). *Journal of the World Aquaculture Society,* **39,** 500-509.

Lunger, A.N., Craig, S.R. & McLean, E. (2006) Replacement of fish meal in cobia (*Rachycentron canadum*) diets using an organically certified protein. *Aquaculture,* **257,** 393-399.

Lunger, A.N., McLean, E., Gaylord, T.G., Kuhn, D. & Craig, S.R. (2007) Taurine supplementation to alternative dietary proteins used in fish meal replacement enhances growth of juvenile cobia (*Rachycentron canadum*). *Aquaculture,* **271,** 401-410.

Lupatsch, I., Kissil, G.W., Sklan, D. & Pfeffer, E. (1997) Apparent digestibility coefficients of feed ingredients and their predictability in compound diets for gilthead seabream, *Sparus aurata* L. *Aquaculture Nutrition,* **3,** 81-89.

Magill, S.H., Thetmeyer, H. & Cromey, C.J. (2006) Settling velocity of faecal pellets of gilthead sea bream (*Sparus aurata* L.) and sea bass (*Dicentrarchus labrax* L.) and sensitivity analysis using measured data in a deposition model. *Aquaculture,* **251,** 295-305.

Maillard, V.M., Boardman, G.D., Nyland, J.E. & Kuhn, D.D. (2005) Water quality and sludge characterization at raceway-system trout farms. *Aquacultural Engineering,* **33,** 271-284.

Malvern (2009) Zeta potential measurement using laser Doppler electrophoresis (LDE). www.malvern.de/LabGer/technology/zeta_potential/zeta_potential_LDE.

MEMG (2004) Marine Environment Quality. Report 1 of 5 Contributions to Charting Progress: An Integrated Assessment of the State of UK Seas, pp. 162.

Milligan, T.G. & Loring, D.H. (1997) The effect of flocculation on the size distributions of bottom sediment in coastal inlets: Implications for contaminant transport. *Water Air and Soil Pollution,* **99,** 33-42.

Mudrak, V.A. (1981) Guidelines for economical commercial fish hatchery wastewater treatment systems In *Fisheries Bioengineering Symposium 1* (Allen, A.J. & Kinney, E.C. eds.), pp. 174 - 182. American Fisheries Society, Bethesda, MD.

Ogunkoya, A.E., Page, G.I., Adewolu, M.A. & Bureau, D.P. (2006) Dietary incorporation of soybean meal and exogenous enzyme cocktail can affect physical characteristics of faecal material egested by rainbow trout (*Oncorhynchus mykiss*). *Aquaculture,* **254,** 466-475.

Olsen, R.E., Suontama, J., Langmyhr, E., Mundheim, H., Ringo, E., Melle, W., Malde, M.K. & Hemre, G.I. (2006) The replacement of fish meal with Antarctic krill, *Euphausia superba* in diets for Atlantic salmon, *Salmo salar*. *Aquaculture Nutrition,* **12,** 280-290.

Olsen, R.E., Hansen, A.C., Rosenlund, G., Hernre, G.I., Mayhew, T.M., Knudsen, D.L., Eroldogan, O.T., Myklebust, R. & Karlsen, O. (2007) Total replacement of fish

meal with plant proteins in diets for Atlantic cod (*Gadus morhua* L.) II - Health aspects. *Aquaculture,* **272,** 612-624.

OSPAR (2006) Background Report on Mariculture (Publication 282/2006).

Panchang, V., Cheng, G. & Newell, C. (1997) Modeling hydrodynamics and aquaculture waste transport in coastal Maine. *Estuaries,* **20,** 14-41.

Patterson, R.N., Watts, K.C. & Timmons, M.B. (1999) The power law in particle size analysis for aquacultural facilities. *Aquacultural Engineering,* **19,** 259-273.

Patterson, R.N. & Watts, K.C. (2003) Micro-particles in recirculating aquaculture systems: particle size analysis of culture water from a commercial Atlantic salmon site. *Aquacultural Engineering,* **28,** 99-113.

Patterson, R.N., Watts, K.C. & Gill, T.A. (2003) Micro-particles in recirculating aquaculture systems: determination of particle density by density gradient centrifugation. *Aquacultural Engineering,* **27,** 105-115.

Pearson, T.H. & Black, K.D. (2001) The environmental impacts of marine fish cage culture In *Environmental Impacts of Aquaculture* (Black, K.D. ed.), pp. 1 - 31. Sheffield Academic Press.

Pereira, T.G. & Oliva-Teles, A. (2002) Preliminary evaluation of pea seed meal in diets for gilthead sea bream (*Sparus aurata*) juveniles. *Aquaculture Research,* **33,** 1183-1189.

Pereira, T.G. & Oliva-Teles, A. (2004) Evaluation of micronized lupin seed meal as an alternative protein source in diets for gilthead sea bream *Sparus aurata* L. juveniles. *Aquaculture Research,* **35,** 828-835.

Pfeiffer, T.J., Osborn, A. & Davis, M. (2008) Particle sieve analysis for determining solids removal efficiency of water treatment components in a recirculating aquaculture system. *Aquacultural Engineering,* **39,** 24-29.

Phillips, M.J., Clarke, R. & Mowat, A. (1993) Phosphorus Leaching from Atlantic Salmon Diets. *Aquacultural Engineering,* **12,** 47-54.

Piedrahita, R.H. (2003) Reducing the potential environmental impact of tank aquaculture effluents through intensification and recirculation. *Aquaculture,* **226,** 35-44.

Pike, I.H. & Barlow, S.M. (2002) Impact of fish farming on fish stocks. *Paper presented at Bordeaux Aquaculture and Environment Symposium,* www.iffo.org.uk/tech/bordeaux.htm.

Pillay, T.V.R. (2004) *Aquaculture and the environment,* Blackwell Publishing.

Pine, H.J., Daniels, W.H., Davis, D.A., Jiang, M.K. & Webster, C.D. (2008) Replacement of fish meal with poultry by-product meal as a protein source in pond-raised sunshine bass, *Morone chrysops* female X *M-saxatlis* male, diets. *Journal of the World Aquaculture Society,* **39,** 586-597.

Pinto, T.A., Colli, C. & Areas, J.A.G. (1997) Effect of processing on iron bioavailability of extruded bovine lung. *Food Chemistry,* **60,** 459-463.

Prakash, K.J., Tyagi, V.K., Kazmi, A.A. & Kumar, A. (2007) Post-treatment of UASB reactor effluent by coagulation and flocculation process. *Environmental Progress,* **26,** 164-168.

Raja, K.B., Simpson, R.J. & Peters, T.J. (1992) Investigation of a role for reduction in ferric iron uptake by mouse duodenum. *Biochimica Et Biophysica Acta,* **1135,** 141-146.

Read, P. & Fernandes, T. (2003) Management of environmental impacts of marine aquaculture in Europe. *Aquaculture,* **226,** 139-163.

Refstie, S., Svihus, B., Shearer, K.D. & Storebakken, T. (1999) Nutrient digestibility in Atlantic salmon and broiler chickens related to viscosity and non-starch

polysaccharide content in different soyabean products. *Animal Feed Science and Technology,* **79,** 331-345.

Refstie, S., Korsoen, O.J., Storebakken, T., Baeverfjord, G., Lein, I. & Roem, A.J. (2000) Differing nutritional responses to dietary soybean meal in rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*). *Aquaculture,* **190,** 49-63.

Refstie, S., Storebakken, T., Baeverfjord, G. & Roem, A.J. (2001) Long-term protein and lipid growth of Atlantic salmon (*Salmo salar*) fed diets with partial replacement of fish meal by soy protein products at medium or high lipid level. *Aquaculture,* **193,** 91-106.

Refstie, S., Landsverk, T., Bakke-McKellep, A.M., Ringo, E., Sundby, A., Shearer, K.D. & Krogdahl, A. (2006) Digestive capacity, intestinal morphology, and microflora of 1-year and 2-year old Atlantic cod (*Gadus morhua*) fed standard or bioprocessed soybean meal. *Aquaculture,* **261,** 269-284.

Regost, C., Arzel, J. & Kaushik, S.J. (1999) Partial or total replacement of fish meal by corn gluten meal in diet for turbot (*Psetta maxima*). *Aquaculture,* **180,** 99-117.

Reid, G.K., Liutkus, M., Robinson, S.M.C., Chopin, T.R., Blair, T., Lander, T., Mullen, J., Page, F. & Moccia, R.D. (2009) A review of the biophysical properties of salmonid faeces: implications for aquaculture waste dispersal models and integrated multi-trophic aquaculture. *Aquaculture Research,* **40,** 257-273.

Robaina, L., Izquierdo, M.S., Moyano, F.J., Socorro, J., Vergara, J.M., Montero, D. & Fernandezpalacios, H. (1995) Soybean And Lupin Seed Meals As Protein Sources In Diets For Gilthead Seabream (*Sparus aurata*) - Nutritional And Histological Implications. *Aquaculture,* **130,** 219-233.

Robaina, L., Corraze, G., Aguirre, P., Blanc, D., Melcion, J.P. & Kaushik, S. (1999) Digestibility, postprandial ammonia excretion and selected plasma metabolites in European sea bass (*Dicentrarchus labrax*) fed pelleted or extruded diets with or without wheat gluten. *Aquaculture,* **179,** 45-56.

Robinson, R.A., and Stokes, R. H. (1955) *Electrolyte solutions, the measurement and interpretation of conductance, chemical potential, and diffusion in solutions of simple electrolytes,* Butterworth Scientific Publications, London.

Romarheim, O.H., Skrede, A., Penn, M., Mydland, L.T., Krogdahl, A. & Storebakken, T. (2008a) Lipid digestibility, bile drainage and development of morphological intestinal changes in rainbow trout (*Oncorhynchus mykiss*) fed diets containing defatted soybean meal. *Aquaculture,* **274,** 329-338.

Romarheim, O.H., Zhang, C., Penn, M., Liu, Y.J., Tian, L.X., Skrede, A., Krogdahl, A. & Storebakken, T. (2008b) Growth and intestinal morphology in cobia (*Rachycentron canadum*) fed extruded diets with two types of soybean meal partly replacing fish meal. *Aquaculture Nutrition,* **14,** 174-180.

Royston, J.P. (1982) An extension of Shapiro and Wilk-W test for normality to large samples. *Applied Statistics-Journal of the Royal Statistical Society Series C,* **31,** 115-124.

Royston, P. (1995) A remark on algorithm AS-181 - the W-test for normality. *Applied Statistics-Journal of the Royal Statistical Society Series C,* **44,** 547-551.

Sabaut, J.J. (2002) Feeding Farmed Fish. *Document presented to the Fisheries Committee of the European Parliament at its Hearing on European Aquaculture,* www.feap.info/production/feeds/sabautcipa_en.asp.

Sanin, F.D. (2002) Effect of solution physical chemistry on the rheological properties of activated sludge. *Water Sa,* **28,** 207-211.

Schneider, O., Amirkolaie, A.K., Vera-Cartas, J., Eding, E.H., Schrama, J.W. & Verreth, J.A.J. (2004) Digestibility, faeces recovery, and related carbon, nitrogen and phosphorus balances of five feed ingredients evaluated as fishmeal alternatives in Nile tilapia, *Oreochromis niloticus* L. *Aquaculture Research,* **35,** 1370-1379.

Schrader, G.A., Zwijnenburg, A. & Wessling, M. (2005) The effect of colloid stability of wastewater treatment plants effluent on nanofiltration performance. *Water Science and Technology,* **52,** 345-357.

Schubert, W. & Gunthert, F.W. (2001) Particle size distribution in effluent of trickling filters and in humus tanks. *Water Research,* **35,** 3993-3997.

Shaw, D.J. (1992) *Introduction to Colloid and Surface Chemistry,* Butterworth Heinemann.

Sherrill, J., Whitaker, B.R. & Wong, G.T.F. (2004) Effects of ozonation on the speciation of dissolved iodine in artificial seawater. *Journal of Zoo and Wildlife Medicine,* **35,** 347-355.

Silvert, W. (1992) Assessing Environmental Impacts of Finfish Aquaculture in Marine Waters. *Aquaculture,* **107,** 67-79.

Silvert, W. & Cromey, C.J. (2001) Modelling impacts In *Environmental Impacts of Aquaculture* (Black, K.D. ed.), pp. 154 - 181. Sheffield Academic Press.

Spinosa, L. & Wichmann, K. (2004) Sludge characterization: the role of physical consistency. *Water Science and Technology,* **49,** 59-65.

Steevens, B. & Sexten, J. (2009) By-Product Feed Price Listing. *University of Missouri Division of Animal Sciences and Commercial Agriculture Program,* agebb.missouri.edu/dairy/byprod/bplist.asp.

Storebakken, T., Kvien, I.S., Shearer, K.D., Grisdale-Helland, B., Helland, S.J. & Berge, G.M. (1998) The apparent digestibility of diets containing fish meal, soybean meal or bacterial meal fed to Atlantic salmon (*Salmo salar*): evaluation of different faecal collection methods. *Aquaculture,* **169,** 195-210.

Storebakken, T., Shearer, K.D., Baeverfjord, G., Nielsen, B.G., Asgard, T., Scott, T. & De Laporte, A. (2000) Digestibility of macronutrients, energy and amino acids,

absorption of elements and absence of intestinal enteritis in Atlantic salmon, *Salmo salar*, fed diets with wheat gluten. *Aquaculture,* **184,** 115-132.

Sugiura, S.H., Dong, F.M., Rathbone, C.K. & Hardy, R.W. (1998) Apparent protein digestibility and mineral availabilities in various feed ingredients for salmonid feeds. *Aquaculture,* **159,** 177-202.

Summerfelt, S.T., Davidson, J.W., Waldrop, T.B., Tsukuda, S.M. & Bebak-Williams, J. (2004) A partial-reuse system for coldwater aquaculture. *Aquacultural Engineering,* **31,** 157-181.

Sutherland, T.F., Martin, A.J. & Levings, C.D. (2001) Characterization of suspended particulate matter surrounding a salmonid net-pen in the Broughton Archipelago, British Columbia. *Ices Journal of Marine Science,* **58,** 404-410.

Tacon, A.G.J. (1997) Fishmeal replacers: Review of antinutrients within oilseeds and pulses - A limiting factor for the aquafeed *Green Revolution*? *Cahiers Options Mediterraneenes - Feeding Tomorrows Fish,* **22,** 153 - 182.

Tacon, A.G.J. & Forster, I.P. (2003) Aquafeeds and the environment: policy implications. *Aquaculture,* **226,** 181-189.

Tacon, A.G.J. & Metian, M. (2008) Global overview on the use of fish meal and fish oil in industrially compounded aquafeeds: Trends and future prospects. *Aquaculture,* **285,** 146-158.

Thapa, K.B., Qi, Y. & Hoadley, A.F.A. (2009) Interaction of polyelectrolyte with digested sewage sludge and lignite in sludge dewatering. *Colloids and Surfaces a-Physicochemical and Engineering Aspects,* **334,** 66-73.

Tibbetts, S.M., Lall, S.P. & Milley, J.E. (2004) Apparent digestibility of common feed ingredients by juvenile haddock, *Melanogrammus aeglefinus* L. *Aquaculture Research,* **35,** 643-651.

Tibbetts, S.M., Milley, J.E. & Lall, S.P. (2006) Apparent protein and energy digestibility of common and alternative feed ingredients by Atlantic cod, *Gadus morhua* (Linnaeus, 1758). *Aquaculture,* **261,** 1314-1327.

Tusek, L., Nitschke, M., Werner, C., Stana-Kleinschek, K. & Ribitsch, V. (2001) Surface characterisation of NH3 plasma treated polyamide 6 foils. *Colloids and Surfaces a-Physicochemical and Engineering Aspects,* **195,** 81-95.

Uran, P.A., Schrama, J.W., Rombout, J., Obach, A., Jensen, L., Koppe, W. & Verreth, J.A.J. (2008) Soybean meal-induced enteritis in Atlantic salmon (*Salmo salar* L.) at different temperatures. *Aquaculture Nutrition,* **14,** 324-330.

Ustaoglu, S. & Rennert, B. (2002) The apparent nutrient digestibility of diets containing fish meal or isolated soy protein in sterlet (*Acipenser ruthenus*). *International Review Of Hydrobiology,* **87,** 577-584.

Valente, L.M.P., Gouveia, A., Rema, P., Matos, J., Gomes, E.F. & Pinto, I.S. (2006) Evaluation of three seaweeds *Gracilaria bursa-pastoris*, *Ulva rigida* and *Gracilaria cornea* as dietary ingredients in European sea bass (*Dicentrarchus labrax*) juveniles. *Aquaculture,* **252,** 85-91.

Valle-Delgado, J.J., Molina-Bolivar, J.A., Galisteo-Gonzalez, F. & Galvez-Ruiz, M.J. (2003) Study of the colloidal stability of an amphoteric latex. *Colloid and Polymer Science,* **281,** 708-715.

Veerapen, J.P., Lowry, B.J. & Couturier, M.F. (2005) Design methodology for the swirl separator. *Aquacultural Engineering,* **33,** 21-45.

Viadero, R.C. & Noblet, J.A. (2002) Membrane filtration for removal of fine solids from aquaculture process water. *Aquacultural Engineering,* **26,** 151-169.

Vogan, C.L., Costa-Ramos, C. & Rowley, A.F. (2001) A histological study of shell disease syndrome in the edible crab *Cancer pagurus*. *Diseases of Aquatic Organisms,* **47,** 209-217.

Walker, A.B., Fournier, H.R., Neffus, C.D., Nardi, G.C. & Berlinsky, D.L. (2009) Partial Replacement of Fish Meal with Laver *Porphyra spp.* in Diets for Atlantic Cod. *North American Journal of Aquaculture,* **71,** 39-45.

Wallace, J. (2008) Zeta potential and particle size characteristics of aquaculture feed ingredients: effects on the physical properties of fish faeces and the efficiency of suspended solid removal in effluent treatment. MSc dissertation, Swansea University.

Walsh, P.J., Blackwelder, P., Gill, K.A., Danulat, E. & Mommsen, T.P. (1991) Carbonate deposits in marine fish intestines - a new source of biomineralisation. *Limnology and Oceanography,* **36,** 1227-1232.

Wang, Y., Guo, J.L., Bureau, D.P. & Cui, Z.H. (2006) Replacement of fish meal by rendered animal protein ingredients in feeds for cuneate drum (*Nibea miichthioides*). *Aquaculture,* **252,** 476-483.

Ward, D.A., Carter, C.G. & Townsend, A.T. (2005) The use of yttrium oxide and the effect of faecal collection timing for determining the apparent digestibility of minerals and trace elements in Atlantic salmon (*Salmo salar*, L.) feeds. *Aquaculture Nutrition,* **11,** 49-59.

Weatherup, R.N. & McCracken, K.J. (1998) Comparison of estimates of digestibility of two diets for rainbow trout, *Oncorhynchus mykiss* (Walbaum), using two markers and two methods of faeces collection. *Aquaculture Research,* **29,** 527-533.

Wong, K.B. & Piedrahita, R.H. (2000) Settling velocity characterization of aquacultural solids. *Aquacultural Engineering,* **21,** 233-246.

Wood, R.J., Stake, P.E., Eiseman, J.H., Shippee, R.L., Wolski, K.E. & Koehn, U. (1978) Effects of Heat and Pressure Processing on Relative Biological Value of Selected Dietary Supplemental Inorganic Iron Salts as Determined by Chick Hemoglobin Repletion Assay. *Journal of Nutrition,* **108,** 1477-1484.

Yamamoto, T. & Akiyama, T. (1995) Effect of Carboxymethylcellulose, Alpha-Starch, and Wheat Gluten Incorporated in Diets as Binders on Growth, Feed-Efficiency, and Digestive Enzyme-Activity of Fingerling Japanese Flounder. *Fisheries Science,* **61,** 309-313.

Ye, L.X., Ritz, D.A., Fenton, G.E. & Lewis, M.E. (1991) Tracing the Influence on Sediments of Organic Waste from a Salmonid Farm Using Stable Isotope Analysis. *Journal of Experimental Marine Biology and Ecology,* **145,** 161-174.

Zhang, Z.-b., Zhao, J.-f., Xia, S.-q., Liu, C.-q. & Wang, X.-j. (2007b) Analysis on mechanism of the chemical-biological flocculation process. *Huanjing Kexue,* **28,** 993-996.

Zhang, Z.B., Zhao, J.F., Xia, S.Q., Liu, C.Q. & Kang, X.S. (2007a) Particle size distribution and removal by a chemical-biological flocculation process. *Journal of Environmental Sciences-China,* **19,** 559-563.

Zhou, Y.N., Xing, X.H., Liu, Z.H., Cui, L.W., Yu, A.F., Feng, Q. & Yang, H.J. (2008) Enhanced coagulation of ferric chloride aided by tannic acid for phosphorus removal from wastewater. *Chemosphere,* **72,** 290-298.

Zouboulis, A.I. & Tzoupanos, N.D. (2009) Polyaluminium silicate chloride-A systematic study for the preparation and application of an efficient coagulant for water or wastewater treatment. *Journal of Hazardous Materials,* **162,** 1379-1389.